CD46-mediated signals in the regulation of T-cell cytokine production and intestinal wound healing

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Abstract

Complement is a key part of innate immunity and vital in the first line of defence against invading pathogens. The understanding of the role of complement in the host's immune defence has undergone its first major change when it became clear that complement is also required for the optimal induction and function of adaptive immunity, particularly B cell activation and T cell effector responses. It is now also becoming increasingly clear that complement plays a key role in the contraction of T cell responses and thus, by definition, in immune homeostasis.

The first part of this thesis focuses on such least understood role of complement. Firstly, evidence is reported for a novel mechanism by which the complement regulatory protein CD46 regulates the key T helper type 1 cytokine IFN-γ and the canonical immunosuppressive cytokine IL-10. The production of both IFN-γ and IL-10 is shown to be temporally regulated by TCR and CD46 signals in $CD4^+$ T cells in an IL-2-dependent fashion and is shown to be defective in rheumatoid arthritis patients.

The molecular pathways linked to the CD46-induced switch of T_H1 cells are largely undefined and were investigated. Gene array studies highlighted the asparagine endopeptidase (AEP) as a candidate molecule involved in the regulation of the switch of T_H1 cells towards IL-10 production. The CD46-driven IFN-γ secretion, but not the intracellular expression, was found to be substantially reduced by AEP inhibition; IL-10 production was consequently affected confirming the importance of IFN-γ signalling in the CD46-induced "switch" mechanism.

The cytokines produced by T cells activated via CD46 may also be important in the crosstalk with surrounding tissues. Here the functional consequences of CD46 signals were further investigated in the context of intestinal epithelial cells to allow the discrimination of the effects of CD46 signals in the two different cells types. A novel role for CD46 in the promotion of cellular proliferation and wound healing was reported.

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I dedicate this thesis to my parents Francesco and Clare, my brothers Giuseppe and Simon and my sister Francesca. Your interest for what I do has always inspired me and you have been great all along the way.

"*Quality needs time*" *Francesco Cardone*

Declaration

I hereby declare that the work included in this thesis is my own work. Wherever contributions of others are involved, every effort has been made to indicate this clearly.

Publications arising from this work

Original publications:

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Invited reviews:

Cardone, J., Le Friec, G., and Kemper, C. (2011). CD46 in innate and adaptive immunity: an update. Clinical and experimental immunology *164*, 301-311.

Cope, A., Le Friec, G., Cardone, J., and Kemper, C. (2011). The Th1 life cycle: molecular control of IFN-gamma to IL-10 switching. Trends in immunology 32, 278-286.

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Abbreviations

Ab	Antibody
AEP	Asparagine endopeptidase
AICD	Activation-induced cell death
APC	Antigen-presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CTLA4	Cytotoxic T⁻lymphocyte antigen 4
DAMPs	Danger associated molecular patterns
DC	Dendritic cell
dH_2O	Deionised water
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
Fab	Fragment, antigen-binding
Fc	Fragment, crystallisable of an antibody
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein 3
FSC	Forward scatter
h	Hour
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunereceptor tyrosine-based activation motif
mAb	Monoclonal antibody
MASPs	MBL associated serine proteases
MBL	Mannose binding lectin
MFI	Geometric mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minutes
mTOR	Mammalian target of rapamycin
PAMPs	Pathogen associated molecular patterns

PBS	Phosphate buffered saline
PE	R-Phycoerythrin
PRR	Pattern recognition receptor
RAPA	Rapamycin
RT	Room temperature
SD	Standard deviation
sec	Seconds
SSC	Side scatter
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
T_H	T helper cell
T-reg	Regulatory T cell
WT	Wild-type

Chapter 1

Introduction

1.1 Interplay between innate and adaptive immunity – an overview

The Nobel Prize in Physiology or Medicine 2011 was divided "with one half jointly to Bruce A. Beutler and Jules A. Hoffmann for their discoveries concerning the activation of innate immunity and the other half to Ralph M. Steinman for his discovery of the dendritic cell and its role in adaptive immunity" (Nobel Prize Press Release, 2011).

Since the 20th century the immune network has been academically and theoretically characterised as dichotomous with the two systems that define it, commonly identified as innate and adaptive immunity. The jointed Nobel Prize awarded in 2011 unifies this text-book definition of immunity highlighting how protection of the host can be fully achieved only as a result of the successful cooperation between innate and adaptive immune responses.

Today we understand that the immune system is essential in order to maintain a healthy homeostatic physiological state. It is necessary in the protection against pathogens but also controls and removes danger associated to injured and transformed tissues and actively takes part to wound healing processes.

The merits of Hoffmann and Beutler rely to the discovery of a key class of pattern recognition receptors (PRRs) involved in the innate immune responses which provide the first line of defence against microbes and modified cells (Lemaitre et al., 1996; Poltorak et al., 1998). They described the functional importance of Toll-like receptor (TLR) 4 in the response to the microbial product lipopolysaccharide (LPS). TLRs are a prime example of germline-encoded receptors able to recognise antigens without any previous exposure. Thanks to PRRs such as TLRs, nucleotide oligomerisation domain-like receptors (NLRs) and lectin like receptors, the innate response is "fast tracking" although not highly specific to the antigen since it recognises highly conserved molecular patterns expressed by classes of microbes. Hence, innate immunity is able to recognise pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) and initiate mechanisms to prevent or eliminate infection and favour wound healing (Akira, 2000). To achieve these complex aims PRRs function in conjunction to cellular components (including granulocytes, mast cells, macrophages and NK cells) and soluble pattern recognition molecules (complement – described in paragraph 1.3-, coagulation, pentraxins).

The evolutionary younger adaptive immune system is a more efficient network in terms of specificity. Furthermore, a key feature of the adaptive responses is immunological memory; subsequential exposure to the same antigen induces a quicker and stronger response (McFarland et al., 2000). The main players of adaptive immunity are represented by lymphocytes; a cell type able to clonally expand in order to generate a highly specific immune response. Lymphocytes are divided in B cells and T cells which have different functions. B cells (named after the Bursa of Fabricius – the organ from which they were discovered in birds) have been initially identified as adaptive cells key in producing pathogen specific antibodies able to recognise antigens in their native form and neutralise pathogens in extracellular compartments (Abbas et al., 2011).

T lymphocytes are a class of immune cells which, unlike B cells, are only able to recognise protein antigens presented by specialised cells and in association with class I or II MHC molecules (Mazza and Malissen, 2007). In 1973 Steinmann was able to identify a novel cell type named dendritic cell (DCs), after the prolonged membrane projections that characterised their morphology (Steinman and Cohn, 1973), critical in eliciting T cell responses. DCs, and more generally antigen presenting cells, are sentinel cells well equipped with co-stimulatory molecules (e.g. namely CD80, CD86 and Notch for DCs) important in T cell development; they express a wide array of PRRs (TLRs, Complement receptors, NLRs, Fc receptors) and have been shown to be key in immune responses since mutations affecting this cell type impairs adaptive immunity. Moreover, impairments influencing an effective trigger and/or development of an immune response can lead to chronic inflammatory- or auto-immune diseases which result from excessive inflammation and uncontrolled immune activation (Hambleton et al., 2011; Rouse and Sehrawat, 2010). The Noble Prize raised the importance of how innate signals (e.g. PRRs) are vital in delivering signals for the development of adaptive immunity.

This requirement of a successful crosstalk between innate and adaptive immunity has been the focus of interest for decades of immunology related research and considerable progress has been made in understanding the underlying mechanisms of danger recognition and adaptive immune instruction. However, the recent notion supporting a role for innate immunity in the contraction of B and/or T cell responses is relatively

novel and not yet broadly accepted. The study developed in this thesis focused on this particular field of immunology and delineated the role of complement-derived innate signals in the induction but particularly the contraction of adaptive human $CD4^+$ T_H1 effector responses.

1.1.1 T lymphocyte development

The thymus represents the main site of maturation of committed lymphoid progenitors which arise in the bone marrow from haematopoietic stem cells (Zuniga-Pflucker, 2004). The different stages of thymocytes development are identified by the expression of surface markers including CD4, CD8, CD25 and CD44 and by the rearrangement status of the T cell receptor (TCR) (Godfrey and Zlotnik, 1993). The first phase of intra-thymic development is known as the double negative (DN) stage 1. Thymocytes in DN1 are identified as $CD4^-/CD8^-/CD25^-/CD44^{HI}$ and have no TCR expression (Godfrey and Zlotnik, 1993; Groettrup and von Boehmer, 1993). Thymocytes in DN1 can differentiate into T, B, natural killer (NK) cells or dendritic cells (DCs) but not into myeloid cells. In the DN2 stage, thymocytes become $CD4^-/CD8^-/CD25^+/CD44^{HI}$ and can only give rise to αβ and γδ T cell lineages or DCs. Subsequently thymocytes become $CD4^-/CD8^-/CD25^+/CD44^{Low}$ in the DN3 stage; in this stage they become committed to the T cell lineage and are able to generate either αβ or γδ T cells (Capone et al., 1998). The loss of CD25 expression in DN4 identifies the pre-stage of double positive (DP) lymphocytes co-expressing CD4 and CD8 (Robey and Fowlkes, 1994). As cells maturate through DN2 and DN4, the rearrangement of TCR β, γ and δ chains is possible. In 90% of the cases, successful TCR rearrangements of the β chain leads to the differentiation of αβ T cells whilst in less than 10% of the cases successful rearrangement of TCR γ and δ anticipates the rearrangements of α and β and generates γδ T cells (Germain, 2002). In the transition between DN4 to double positive (DP) T cells, the αβ $CD4^+CD8^+$ cells undergo proliferation and subsequently interact with MHC class I and class II associated self-peptides presented by thymic epithelial cells (Rothenberg and Dionne, 2002). The nature of the MHC molecule (class I vs. class II) and the strength of the TCR signals will determine whether a DP cell will become single positive for CD4 or CD8; only intermediate levels of signalling will translate in the positive selection of T cells (Izon et al., 2001). A pivotal role in fate decision

during T cell development has been also described for the Notch pathway (Anderson et al., 2001). In fact, the interaction between Notch receptor-expressing thymocytes and Notch ligand-expressing thymic stromal cells, triggers key signals during the T-cell maturation process promoting the commitment towards the αβ T cell lineage inducing the generation of either $CD4^+$ T cells or $CD8^+$ cytotoxic T cells (Robey et al., 1994).

1.1.2 αβ T cells

Mature T cells that leave the thymus may be already lineage committed as in the case of natural regulatory T cells or may still not be functionally differentiated (Maggi et al., 2005; Zuniga-Pflucker, 2004); in the latter case they are known as naïve T lymphocytes because although mature they have not encountered the antigen they are specific for. Once they leave the thymus naïve T cells re-circulate through the blood and lymph in a resting state. The activation process of T cells into an effector phenotype requires the input of several signals; signal 1 (T cell receptor engagement), signal 2 (costimulation) and signal 3 (cytokine receptor engagement). These three signals are delivered generally by activated antigen presenting cells (APCs) because they express peptide-loaded MHC (pMHC) class I and II molecules required for T cell receptor (TCR) engagement, ligands for T cell costimulation and T cell effector function-defining cytokines (Croft, 2003). The interaction between T cells and APC is anatomically confined to specialised lymphoid tissues known as secondary lymphoid organs (Bousso, 2008; Steinman and Cohn, 1973). These include the spleen and lymph nodes, and represent the tissues to which protein antigens are shuttled once they are captured by APCs at the level of skin, gut and lungs (Abbas et al., 2011).

1.1.3 Mechanisms and induction of effector $CD4^+$ T cells

1.1.4 TCR signalling (Signal 1)

The αβ TCR is a heterodimer composed of two transmembrane polypeptides known as α and β (Figure 1.1). Both α and β are composed of an amino-terminal variable region responsible for the recognition of the antigen peptide and a carboxi–terminal constant region non-covalently associated to signalling proteins known as the CD3 complex and

ζ (Figure 1.1) (Bentley et al., 1995; Fields et al., 1995; van der Merwe and Dushek, 2011). The TCR interaction with the pMHC is the first step in triggering T cell activation (Signal 1). Key in the process of TCR-pMHC interaction is the role of co-receptors CD4 and CD8 (Rudolph et al., 2006). The CD4 co-receptor interacts with non-polymorphic regions of the MHC class II molecule driving T cell immunity towards antigens captured from the extracellular space (i.e. parasites and extracellular bacteria) while MHC class I will present cytosolic antigens (i.e. from viruses or cancer cells) that specify for a CD8 T cell response (Abbas et al., 2011). Sufficient engagement of MHC-peptide/TCR complexes leads to the formation of the immunological synapse (IS) as a platform for subsequent signalling events required for T cell activation (Fooksman et al., 2010).

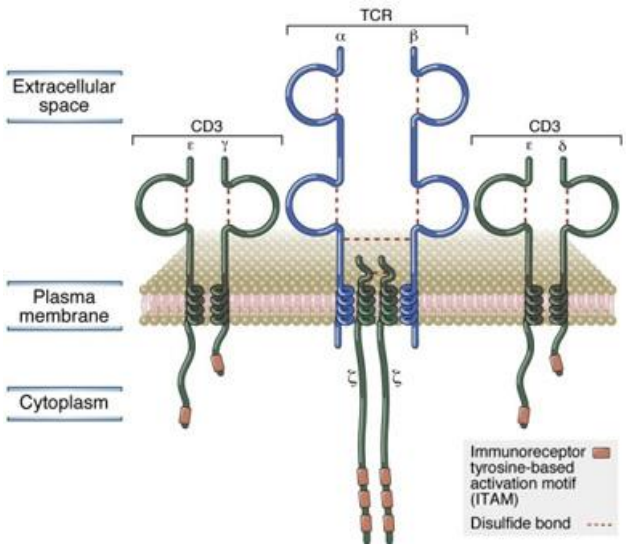


Figure 1.1 Structure of the T cell receptor complex.
The αβ TCR is a heterodimer which comprises two chains; α and β. The two chains are associated through a disulphide bridge ad stabilised by intra-disulphide bridges. The carboxy–terminal constant region is non-covalently associated to signalling proteins on the intracellular region known as the CD3 complex and ζ. The three different subunits of CD3 γ, δ and ε, form heterodimers and comprehend one immunereceptor tyrosine-based activation motif (Nagasawa et al.) (Nagasawa et al.) each. The ζ signalling molecule forms homodimers that associate with the TCR and each ζ is characterised by the presence of three ITAMs (Abbas et al., 2011).

At the level of the IS segregation of TCR, CD3, CD4 or CD8 and CD28 from CD45 and other inhibitory molecules takes place and allows for T cell activation (Dustin and Depoil, 2011). Several signalling pathways activated by the TCR engagement are required for the initiation of cell cycle progression and the production of the key T cell growth factor IL-2; additional incoming signals from co-stimulators and cytokine

receptors will subsequently complement TCR signals and define the T cell effector functions.

Antigen recognition and CD4/CD8 co-receptor engagement induce different T cell signalling pathways; the mostly studied are the mitogen activated protein (MAP) kinase pathway and the phospholipase C-γ1 (PLC-γ1) pathway.

MAPK signalling is activated through the phosphorylation of CD3 and ζ by adaptor molecule LAT (Figure 1.2). LAT regulates the guanine nucleotide exchange factor son of sevenless (Sos) that activates Ras (a GTPase linked to lipids of the cell membrane) (Abraham and Weiss, 2004). Activated Ras triggers the extracellular signal regulated kinase (Wingender et al.) cascade by inducing phosphorylation of ERK1 and ERK2. This results in the activation of c-Fos, a transcriptional regulator of activator protein 1 (AP1). AP1 is a transcription factor particularly important in the synthesis of IL-2 the key growth cytokine produced by T cells (Smith-Garvin et al., 2009).

TCR induced activation of the adaptor molecule LAT (Figure 1.2) also promotes the recruitment of phospholipase C-γ1 (PLC-γ1). Activated PLC-γ1 hydrolyses phosphatidyl inositol-4,5 biphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (Smith et al.). IP_3 is a second messenger that induces increases in Ca^{2+} intracellular levels. Calcium acts as a signalling molecule activating calmodulin, an important serine threonine phosphatase that activates transcription factors including the nuclear factor of activated T cell (NFAT). NFAT acts in conjunction with AP1 and is pivotal in the regulation of genes such as IL-4 and IL-2 hence in the regulation of T_H1 and T_H2 responses (Hermann-Kleiter and Baier, 2010). DAG is able to activate protein kinase C-θ (PKCθ) and activate NF-kB, a crucial transcription factor for the production of cytokines (e.g. IL-2, IFN-γ and IL-12) (Abraham and Weiss, 2004).

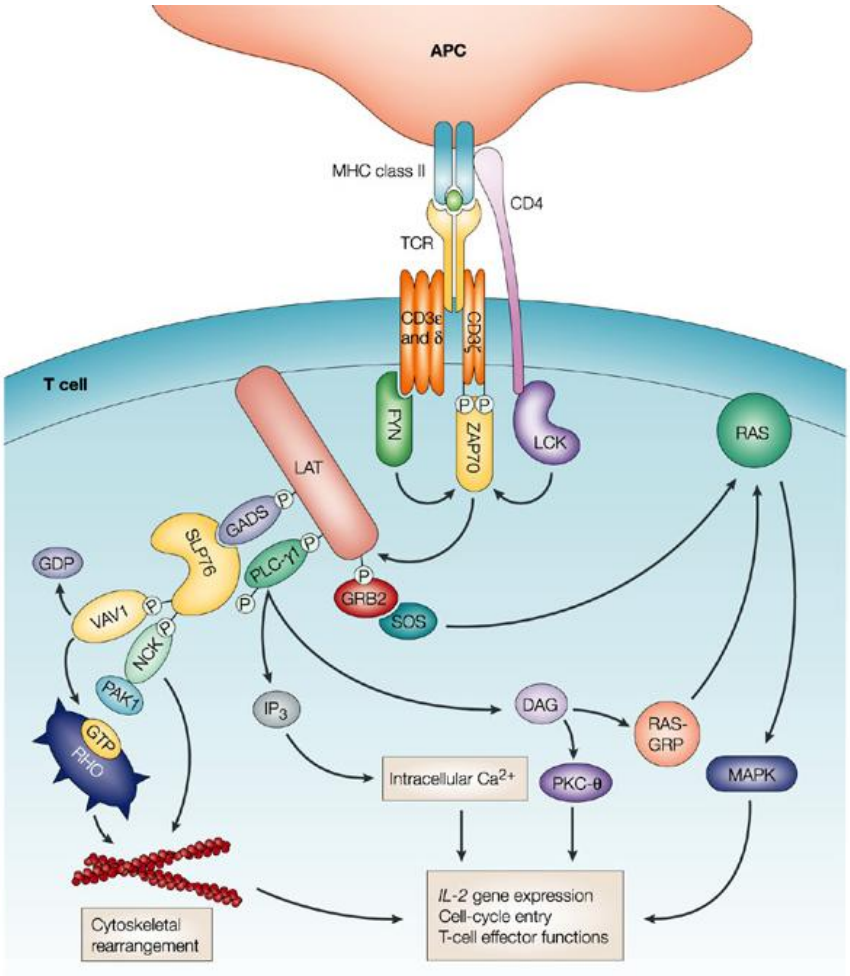


Figure 1.2 TCR activation and signalling.
TCR signalling is only initiated when the TCR stably interacts with the peptide associated to MHC molecules. Once the TCR has stably interacted with the pMHC, the tyrosines present in the ITAMs of CD3 and ζ are phosphorylated by Lck and Fyn kinases which are in an active state due to the removal of phosphogroups by the phosphatase CD45. The phosphorylation of ITAMs at the level of TCR ζ favours the interaction with a tyrosine kinase known as zeta associated protein 70 (ZAP70); the bound ZAP70 is phosphorylated by Lck. As a result ZAP70 initiates PLC-γ1 and MAP kinases pathways through adaptor molecules such as LAT and VAV which promote the production of second messengers including inositol 3,4,5-triphosphate (IP_3) and diacylglycerol (Smith et al.). IP_3 can induce an increase in Ca^{2+} concentration whilst DAG can in turn activate RAS guanyl nucleotide-releasing protein (RASGRP) (Abraham and Weiss, 2004).

1.1.5 Co-stimulatory signals for $CD4^+$ T cells (Signal 2)

In order to achieve complete T cell activation, TCR triggered signals need to be complemented by signalling of co-stimulatory molecules. To date several positive co-stimulators of T cell activation have been identified including CD2, CD28, CD30 CD40L, inducible T cell co-stimulator (Hutloff et al., 1999) and OX40 (Croft, 2003). However, the best characterised co-stimulator is CD28. CD28 is a homodimer with a single Ig extracellular domain (Kovacs et al., 2005). It is expressed on 90% of $CD4^+$ human T cells and its physiological activation is triggered by the APC expressed CD28 ligands, CD80 (B7-1) and CD86 (B7-2) (Riley and June, 2005). PRRs (TLRs or NLRs or LLRs) engagement, complement opsonised antigens and cytokine signalling increase the expression of MHCII, CD80 and CD86 molecules favouring APCs maturation (Carroll, 2004). Lack of CD28 co-stimulation has been shown to impair T cells responses towards infecting pathogens highlighting the importance of positive costimulation in T cell activation and skewing (Jankovic et al., 2001; Shahinian et al., 1993). In conjunction with TCR engagement, CD28 contributes to T cell activation through enhancement of tyrosine phosphorylation events (Michel et al., 2000). Upon CD28 engagement, the intracellular tail of CD28 associates with the PI3 kinase activating PLC-γ1, PKCθ and MAPK pathways (Ueda et al., 1995) inducing cellular proliferation and IFN-γ, IL-2 and TNF production (Thompson et al., 1989). As a result of these events CD28 positively regulates T cells proliferation, differentiation and cytokine secretion. CD28 is the canonical example of a positive T cell co-stimulatory molecule; however negative co-stimulatory molecules are necessary to assure the appropriate contraction of T effector responses. To date several negative co-stimulators of T cell proliferation/activation have been described including Cytotoxic T-lymphocyte antigen 4 (CTLA4), T cell immunoglobulin mucin-(TIM)-3 and programmed cell death (Figure 1.3) (Acuto and Michel, 2003; Tivol et al., 1995; Waterhouse et al., 1995; Zakrzewski et al., 2006). T cell co-stimulatory molecules bare high potential for therapeutic applications. Noteworthy is the development of a CTLA4-Ig protein (Abatacept) which has been successfully used for the treatment of rheumatoid arthritis (Ruderman and Pope, 2005).

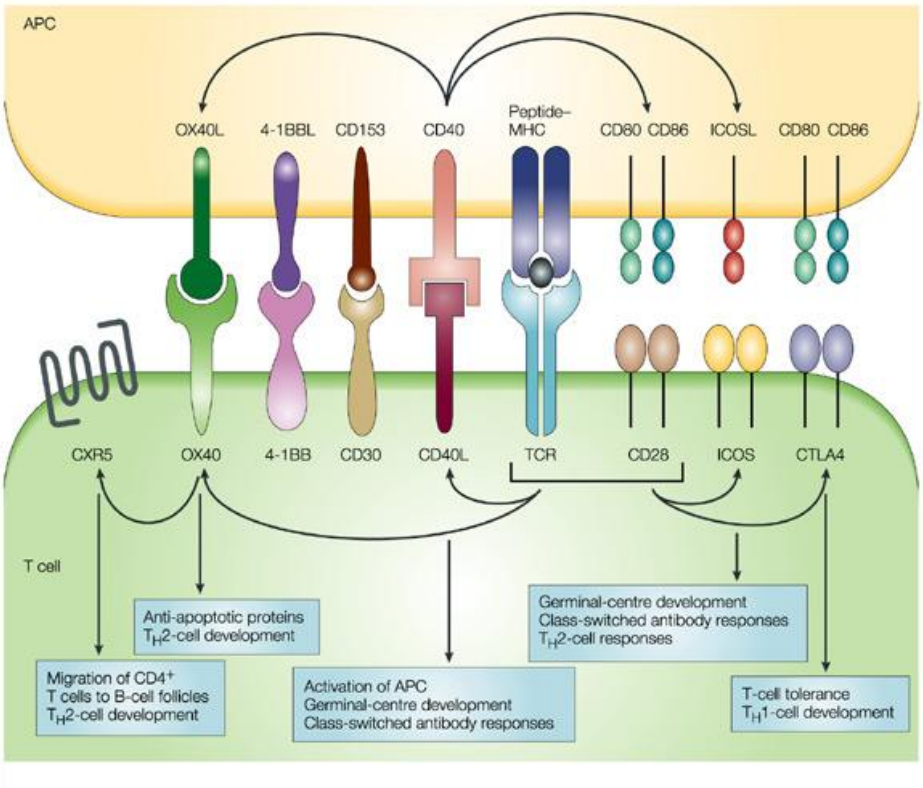


Figure 1.3 TCR co-stimulatory molecules
CD28 and CTLA4 are two examples of "second signal" co-stimulatory molecules with positive and negative properties respectively. CD40 ligand (CD40L) expression is induced during antigen recognition by T cells. The interaction with its receptor CD40 expressed on the APC induces increased expression of B7 molecules CD80, CD86 and OX40 ligand (OX40L) favouring T cell activation. Expression of inducible co-stimulatory molecule ligand (ICOSL) by activated APCs (including DCs and B cells) has been shown important in T_H2 induced antibody production and in the development of follicular T helper cells (Acuto and Michel, 2003).

1.1.6 Induction of effector functions via cytokines (Signal 3)

The magnitude and specificity of signals 1 and 2 are pivotal in the initiation of T cell lineages decisions. However, T cell subset-instructing cytokines which trigger signal 3 ultimately define the effector function of an activated T cell (Croft, 2003). T cells can differentiate in several subsets each specific for classes of pathogens and characterised by signature transcription factors and secreted cytokines. The publication on the Journal of Immunology in 1986 of Tim Mosmann and Bob Coffman (Mosmann et al., 1986) was the first to identify two main mouse $CD4^+$ T cell subsets known as Type 1 T helper cells (T_H1), characterised by IFN-γ production and Type 2 T helper cells (T_H2), characterised by IL-4 production. In the 1990s, Susan Swain started pivotal experiments in the race to decipher T helper subsets. She showed that naïve T cells could only produce IL-4 in the presence of TCR stimulation and IL-4 receptor signalling (Swain et al., 1990). Murphy's group in 1993 showed that it was possible to induce T_H1 cells through macrophage derived IL-12 (Hsieh et al., 1993). In 1994 a "tolerant" T cell subtype was identified by Weiner. This cell subpopulation was called Th3 and was characterised by the production of immunosuppressive TGF-β (Ehret et al., 2001). In 2003 Rudensky and Sakaguchi's groups revolutionised immunological research focused on tolerance through the identification of the key transcription factor of regulatory T cells; Foxp3. The *in vivo* relevance of these findings was further confirmed with the identification that the human immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome was associated with mutations in Foxp3 (Hori et al., 2003; Patel, 2001; Wildin et al., 2001). With the insight of these studies immunologists recognise now an increasing number of T cell subsets. The identification of novel T cell subsets and cytokines is an active field of research, and a novel concept of T cell plasticity by which specific T helper subsets can revert their phenotype has been proposed and proved (Murphy and Stockinger, 2010). Recently T cells secreting abundant IL-9 (Dardalhon et al., 2008; Veldhoen et al., 2008) and IL-22 (Trifari et al., 2009; Veldhoen et al., 2008) have been identified and named T_H9 and T_H22 respectively. However, since no transcriptomic signature has been associated to such subsets, the following paragraphs will only focus on the main characteristics of T_H1, T_H2, and T_H 17 effector cells.

1.1.7 T helper 1 lymphocytes - T_H1

T_H1 cells provide the cell-mediated immune response against intracellular bacteria including listeria, salmonella and mycobacteria and to intracellular parasites such as leishmania (Hultgren et al., 2004; Ravindran et al., 2005; Sullivan et al., 2005; Szabo et al., 2000). They are also vital in the activation of cytotoxic $CD8^+$ T cells fighting viral infections (Actor et al., 1994; Shirai et al., 1998). The direct infection of DCs and/or macrophages or the engagement of germ-line encoded PRR (i.e. TLRs or NLRs) with intracellular pathogen antigens, triggers the APCs to produce IL-12. The IL-12 receptor comprises the IL12Rβ1 chain (Figure 1.4) which is expressed on all naïve T cells and the IL-12Rβ2 chain which is induced by TCR activation and maintained by IL-12 (Afkarian et al., 2002; Moon et al., 1997). In conjunction with TCR and CD28, IL-12 signalling induces phosphorylation of the signal transducer and activator of transcription (STAT) 4 leading to the synthesis of IFN-γ, the key effector cytokine for T_H1 lymphocytes (Kaplan et al., 1996b). IFN-γ production by T_H1 is not only functionally important for macrophage activation and DCs maturation during pathogen clearance; it is also essential to enhance the positive feedback loop for T_H1 skewing, by inducing the STAT1 through the IFN-γ receptor (Afkarian et al., 2002). Furthermore, it promotes the inhibition of the T_H2 and T_H17 subsets. The main mechanism of such process is governed by T-bet, the key transcription factor associated to T_H1 differentiation (Szabo et al., 2000). T-bet is a T-box transcription factor able to bind and promote transcription of the *Ifng* gene whilst inhibiting IL-4 transcription (crucial in T_H2 development) and preventing activation of the *RORγt* gene (essential for T_H17 skewing) (Doffinger et al., 2002). The *in vivo* relevance of T_H1 cells is clear; mice with defects in T_H1 induction (i.e. T-bet knockout mice) are unable to clear infection of intracellular pathogens including L. major, M. tuberculosis or S. typhimurium. Further such animals are susceptible to typical T_H2 diseases such as asthma (Ravindran et al., 2005; Sullivan et al., 2005; Szabo et al., 2000). In a similar way, mutations in the IL-12 or IFN-γ-induced T-bet pathway lead to T_H1 exaggerated induction and have been associated to autoimmune disorders such as type 1 diabetes (Hultgren et al., 2004; Manoury-Schwartz et al., 1997; Sasaki et al., 2004).

1.1.8 T helper 2 cells - T_H2

T_H2 lymphocytes are essential in fighting infections against helminths and in developing a response to allergens. T_H2 cells are defined by the expression of the key transcription factor GATA-3 (Figure 1.4) and by the signature cytokines IL-4, IL-5 and IL-13 (Glimcher and Murphy, 2000). The main effector functions of T_H2 cells include induction of IgE production in B cells (Kopf et al., 1993; Ueda et al., 1990), alternative macrophage activation through IL-4/13 production and recruitment of eosinophils through IL-5 secretion (Coffman and Carty, 1986; Gordon, 2003). The initial source of the T_H2 driving IL-4 has been enigmatic for a long time as APCs do not produce this cytokine. Initially it was suggested that the basal IL-4 production induced in the presence of a T_H promoting environment and the IL-4 positive feedback loop could be sufficient in enhancing and directing T_H2 skewing (Kaplan et al., 1996a; Swain et al., 1990; Zheng and Flavell, 1997). However, to date other sources of IL-4 have been identified including mast cells and basophils, which could be supporting *in vivo* T_H2 induction (Paul, 2010). An important role for T_H2 unwanted induction against innocuous antigens in asthma has been demonstrated and a correlation of disease severity with frequencies of T_H2 cells and cytokines highlights the importance of such subset in human disease (Robinson et al., 1992).

1.1.9 T helper 17 cells - T_H17

T_H17 cells are defined by the production of IL-17a and were described in 2005 for the first time (Bendelac et al., 1997). T_H17 cells are important in mediating immunity towards extracellular bacteria including gram-positive (e.g. *Propionibacterium acnes*) and gram-negative species (e.g. *Citrobacter rodentium, Klebsiella pneumonia*) and towards fungi (Conti et al., 2009; Itoh et al., 1991; Miossec et al., 2009). The differentiation of T_H17 was initially described as dependent on IL-6 and TGF-β; these cytokines induced the transcription factor RORγt (Figure 1.4) which has since then been defined as the master regulator of T_H17 cells (Ivanov et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). T_H17 cells have been shown to be able to secrete an array of cytokines with contrasting functions including: IL-17a, IL-17f, IL-21, and IL-22. IL-17a and IL-17f are genetically linked and often co-synthesized. They both bind to

the IL17 receptor A (IL-17RA) although IL-17a has higher affinity. IL-17a and IL-17f can activate neutrophils inducing active responses towards fungi and extracellular bacteria (Ferretti et al., 2003). IL-17a can induce the production of the pro-inflammatory cytokine IL-6 and the chemotactic interleukin IL-8. IL-21 produced by T_H17 cells is a stimulatory factor for T_H17 differentiation and serves as the positive feedback amplifier (Korn et al., 2007). Importantly, mutations in the signal transducer STAT3 which is important in T_H17 differentiation are associated with recurrent fungal and bacterial infections further attesting to the importance of this subset of T_H cells *in vivo* (Milner et al., 2008).

A prominent role for T_H17 in autoimmune disease has also been proven. Initial observations showed that IL-17a deficiency was protective in a model of experimental autoimmune encephalomyelitis (Andreae et al.), which resembles human multiple sclerosis (Komiyama et al., 2006). A subsequent study by Murphy's group clarified that the pathogenic effect in EAE was caused by the cytokine IL-23 (Cua et al., 2003). Further, the effect was not mediated by T_H17-derived IL-22 since IL-22 knockout mice are not protected from EAE (Kreymborg et al., 2007). A role for T_H17 cells has been suggested in the autoimmune disorder rheumatoid arthritis (Hirota et al., 2007).

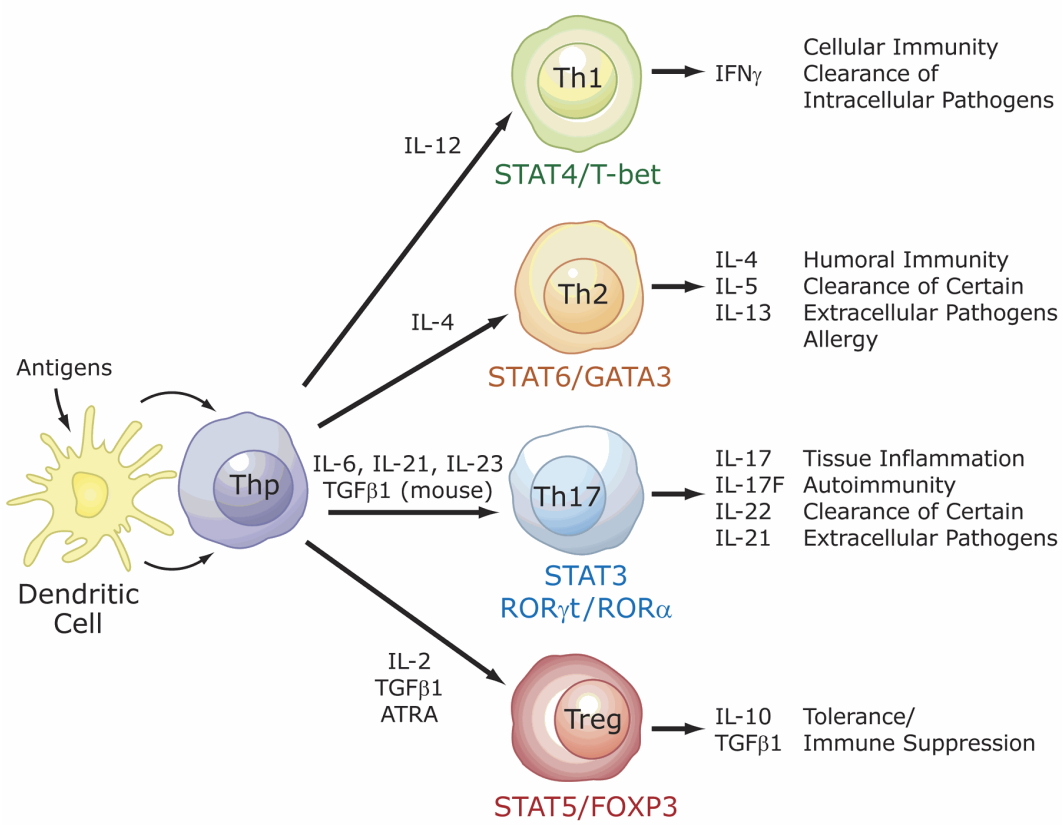


Figure 1.4 T helper cell subsets.
The differentiation of $CD4^+$ T cells into a specific lineage initiates upon cognate interaction between Naïve $CD4^+$ T_H cells (Thp) and APCs which provides Signal 1 (TCR) and Signal 2 (CD28/ICOS). The cytokine milieu determines the lineage commitment and effector function of T_H cells (Signal 3). IL-12 induces the activation of STAT4 signalling promoting production of IFN-γ. Signalling *via* the IFN-γ receptor induces STAT1 and transcription factor T-bet. As a result the expression of IL-12Rβ2 and IFN-γ are increased and promote T_H1 skewing. IL-4 induces STAT6 activation and consequently the expression of the transcription factor GATA-3 which directs T_H2 cell lineage differentiation and suppresses T_H1 skewing. T_H17 differentiation can be induced by different cytokines; IL-6 and TGF-β1 as well as IL-21 and IL-23 receptor engagement are pivotal in promoting the expression of the lineage transcription factor RORγt. High environmental IL-2 is a requirement for the induction of STAT5 signalling and Foxp3 expression in natural regulatory T cells (Jetten et al., 2009).

1.1.10 $CD8^+$ T cells

$CD8^+$ T cells recognise peptide antigens presented in association with MHC class I molecules (Russell and Ley, 2002). CD28 costimulation, through APCs expressing CD80 and CD86, and signalling of inflammatory cytokines, mainly through IL-12 and type I interferons, promote naïve $CD8^+$ T cells proliferation and differentiation into cytotoxic T lymphocytes (CTLs) (Cui et al., 2009; Curtsinger et al., 2005). CTLs recognise and kill infected cells that express the MHC class I associated antigen, which triggers the differentiation of the naïve $CD8^+$ T cells. Two main mechanisms of target cell killing by $CD8^+$ T cells have been described. The granule-dependent mechanism relies on the targeted delivery of CTLs granules containing perforin and granzymes to the point of contact with the target cell (Catalfamo and Henkart, 2003). Perforin, a homologue of complement component C9, forms pores in the target membrane and facilitates the release of granzymes in the cytosol of the target cell allowing the initiation of apoptosis (Persechini et al., 1992). A second mechanism of CTLs target killing is granule-independent and relies on the binding of Fas (CD95), expressed on the target cell, by the Fas ligand which also results in the activation of caspase enzymes and ultimately apoptosis of the target cells (Delves and Roitt, 2000; Itoh et al., 1991; Yonehara et al., 1989). CTLs have the capacity to secrete cytokines including IFN-γ and TNF-α which promote phagocytic activity of macrophages and increase the expression of MHC class I on virus-infected cells for enhanced recognition of viral peptides (Mosmann et al., 1997). Studies on the role of $CD4^+$ T cells in $CD8^+$ T cell-mediated responses have highlighted the ability of $CD4^+$ T cells to provide help for $CD8^+$ activation directly via the secretion of cytokines or indirectly by potentiating APCs function (e.g. through the CD40/CD40L axis) (Schoenberger et al., 1998). Although this mechanism seemed to be specific according to the infecting pathogen latest findings support the theory that $CD4^+$ T cells provide help for most CTL responses and that they are key in providing signals for effective secondary response and differentiation into memory $CD8^+$ T cells (Janssen et al., 2003; von Herrath et al., 1996).

1.1.11 γδ T cells

γδ T cells represent a subset of "non-conventional" T cells which, unlike αβ T cells, do not recognize MHC associated peptide antigens. Similarly to the αβ TCR, the γδ TCR is associated to CD3 and ζ proteins and its engagement induces the signalling events described above (paragraph 1.1.4). γδ T cells are mainly distributed in epithelial surfaces and in the mucosa of the respiratory, digestive and reproductive systems (Hahn et al., 2004; Inagaki-Ohara et al., 2005; Lewis et al., 2006). Extensive studies on the antigens recognised by γδ T cells have shown that this particular subset of T cells responds to a variety of stress induced molecules, DAMPs and PAMPs. Ligands for the γδ TCR comprise MHC related molecules (e.g. CD1c molecules) and MHC class I unrelated molecules such as viral glycoproteins, lipids and phospho-antigens (Hayday, 2009). However, a key feature of γδ T cells is the recognition of antigens through non-clonal receptors but rather through natural killer receptors (NKR) and TLRs. The natural killer group 2, member D (NKG2D) receptor has been highly investigated (Groh et al., 1998). NKG2D, which is broadly expressed by γδ T cells, is a lectin-like receptor which recognises MHC class I related ligands usually up-regulated on transformed or virally infected cells (Eagle and Trowsdale, 2007). NKG2D-mediated signals have been shown to trigger the cytotoxic activity of γδ T cells and to function in conjunction with γδ TCR activation through the retinoic acid early transcript 1 (RAE1) and the MHC class polypeptide-related sequence A (MICA) (Groh et al., 1999).

The effector mechanisms of γδ T cells include the killing of infected or transformed cells via the engagement of FAS or TNF-related apoptosis-inducing ligand receptors (Dalton et al., 2004). The cytotoxic activity of γδ T cells may be also mediated by the secretion of perforins and granzymes (e.g. granzyme B) which are up-regulated upon activation of γδ T cells (Fahrer et al., 2001; Vermijlen et al., 2007). Furthermore, γδ T cells have been shown to secrete a wide array of cytokines including TNF, IFN-γ, IL-4, IL-5, IL-13 and IL-17 promoting T helper responses in the host defence against intracellular and extracellular pathogens (Hahn et al., 2004; Roark et al., 2007; Tagawa et al., 2004). Importantly, although γδ T cells have strong inflammatory and cytotoxic properties, it has been shown that they also express epithelial growth factors, which promote processes of wound-healing (Jameson and Havran, 2007; Toulon et al., 2009).

1.1.12 NKT cells

NKT cells represent a population of αβ T cells with limited diversity within the α chain of the αβ TCR (Borg et al., 2007). They express typical NK markers such as CD56 and are particularly abundant in the liver and bone marrow where they represent between 30-50% and 20-30% of mature mouse T cells respectively (Bendelac et al., 1997). NKT cells are specific for glycolipid antigens presented by MHC class I-like molecules such as CD1d (Benlagha et al., 2000). NKT cells can produce cytokines with distinct downstream effects reflecting the existence of different NKT subtypes. $CD4^+$ NKT cells have been shown to produce T_H1 and T_H2 cytokines while $CD4^-$ NKT cells mainly produce T_H1 cytokines (Doherty et al., 1999). Several studies have shown that NKT cells are able to secrete cytokines rapidly and it has been suggested that they have a role in promoting marginal zone B cells to produce antibodies against lipid antigens (e.g. mycobacterial antigens) (Chang et al., 2011). Environmental cytokines can influence responses of NKT cells; for instance IL-12 has been show to promote IFN-γ production (Leite-De-Moraes et al., 1998). The effector functions of NKT cells include cytolytic activity via the Fas ligand, which is constitutively expressed on NKT cells and can target Fas expressing targets, or via perforin-dependent mechanisms (Arase et al., 1994; Smyth et al., 2000). The role of NKT cells in human disease remains a matter of investigation. Thus far, roles for NKT cells in infection, mainly mycobacteria, and cancer rejection have been shown (Grant et al., 1999; Nakagawa et al., 1998). Further elucidation of the mechanisms involved will shed light on the *in vivo* importance of this T cell subset.

1.2 Immunological Tolerance

The ability of the immune system to clear infecting pathogens and then initiate the required tissue repair at the site of infection is dependent on a fine balance between effector and regulatory mechanisms. Central in maintaining the functionality of the immune system is its ability to induce tolerance towards self or innocuous antigens (e.g. commensals) (Sakaguchi et al., 2008). The concept of immunological tolerance refers to the ability of the immune system to remain unresponsive to the presence of innocuous antigens; this is critically important at host-environmental interfaces (i.e. gut, skin and lungs) where there is continuous exposure to danger (invading pathogens) and non-danger (commensals, food) signals. Impairment to maintain tolerance towards non-dangerous (self) antigens translates into pathological conditions including hypersensitivity diseases, IBD, asthma, allergy and autoimmune diseases including rheumatoid arthritis, multiple sclerosis (Astier et al., 2006; Coombes et al., 2005; Hawrylowicz and O'Garra, 2005; Kearley et al., 2005). Central tolerance refers to the mechanisms that allow for the discrimination and removal of high and low avidity self-reactive lymphocytes in the generative lymphoid tissues (e.g. thymus and bone marrow)(Hogquist et al., 2005). Peripheral tolerance refers to the immunological mechanisms that are in place to control self-reactive T cells, which have escaped central tolerance and have migrated into peripheral tissues (Vignali et al., 2008). Auto-reactive T cells in the periphery may be suppressed in three main ways including, anergy (functional unresponsiveness), activation induced cell death and suppression *via* regulatory T cells or tolerogenic DCs (Mazzucchelli et al., 1996).

The following paragraphs will focus on the mechanisms of immunological tolerance mediated by $CD4^+$ regulatory T cells, since these are most pertinent to the studies related to this thesis.

1.2.1 Natural T-regulatory cells in peripheral tolerance

T regulatory lymphocytes (T-regs) are pivotal in order to maintain immune homeostasis at the host/environment interface and limit immunopathologies during inflammatory reactions (Sakaguchi et al., 2008). Currently, two major T-reg populations are recognised. The first group, natural T-regs are thymus-derived and pivotal in suppressing self-reactive effector T cells. They are characterized by high CD25 expression, the requirement of high environmental IL-2 for their expansion (Sakaguchi et al., 1995) and the expression of the forkhead transcription factor Foxp3 (Hori et al., 2003). Mutations in the *Foxp3* gene are associated with the immune dysregulation, poliendocrinopathy, entheropathy (IPEX) syndrome (Bennett et al., 2001). Children affected by IPEX develop diabetes mellitus, enteropathy, eczema and severe allergies as a consequence of an altered function of natural T-regs stressing their *in vivo* importance in immune regulation (Caudy et al., 2007; van der Vliet and Nieuwenhuis, 2007). Several mechanisms have been proposed for the suppression of effector T cells by natural T-regs. Cell contact-independent mechanisms of suppression (Figure 1.5) include secretion of immunosuppressive cytokines such as TGF-β, IL-10 and IL-35 and IL-2 (Vignali et al., 2008). Cell/cell contact-dependent mechanisms of suppression include perforin- or granzyme-secretion and delivery of negative signals in the responder cell such as cAMP (Sakaguchi et al., 2008). Natural T-regs may also suppress T cell responses by preventing dendritic cell (DC) maturation and function *via* down regulation of CD80, CD86 and MHCII expression.

1.2.2 Adaptive T regulatory cells in peripheral tolerance

The second group of T-regs is known as adaptive T-regs and they are thought to mediate tolerance towards non-self antigens specifically at the host/environment boarders such as the gut, skin and lung (Roncarolo et al., 2001; Rubtsov et al., 2008). Diverse subsets of adaptive T-regs have been described including type 1 regulatory T cells (Tr1) which are IL-10 producing (Groux et al., 1997) and Th3 cells (TGF-β producing) (Carrier et al., 2007; Ehret et al., 2001). However, specific conditions are required to obtain each subset of adaptive T-regs *in vitro* and unlike the natural T-regs there is no unique marker to enable their identification *ex-vivo* (Roncarolo et al., 2006).

Although the *in vivo* functionality has not been ascertained, it is well established that the main common feature of adaptive T-regs is their ability to suppress T effector cells through the secretion of the canonical immunosuppressive cytokine IL-10 (Battaglia et al., 2006b; Roncarolo et al., 2006). In line with this is the fact that IL-10-deficient mice or humans with mutations in the IL-10 receptor suffer from colitis (Kuhn et al., 1993; Ziegler-Heitbrock et al., 2003). Although the induction/expansion of IL-10-secreting adaptive regulatory T cells has been proven using biological molecules including retinoic acid (Mucida et al., 2009), vitamin D3 (Xystrakis et al., 2006) and chemical compounds such as rapamycin (Battaglia et al., 2006a; Battaglia et al., 2006c) there is still a massive lack in the understanding of their induction and regulation pathways. This is clearly a void that needs to be filled as these cells may bear high potential to be used/targeted therapeutically in the settings of transplantation or autoimmunity. A current question relevant to the field is whether IL-10-producing T-regs constitute a separate lineage or whether they are rather a specific 'phase' during the life cycle of an effector T cell response (Cope et al., 2011).The latter notion is lately gaining weight among scientists, particularly because it has been observed that the main effector T cell subpopulations (T_H1, T_H2 and T_H17) can secrete IL-10 when activated under appropriate conditions (McGeachy et al., 2007). The work in this thesis further contributes to this understanding and demonstrates that such particular signals (here CD46) can indeed induce IL-10 in human T_H1 cells and confer regulative activity.

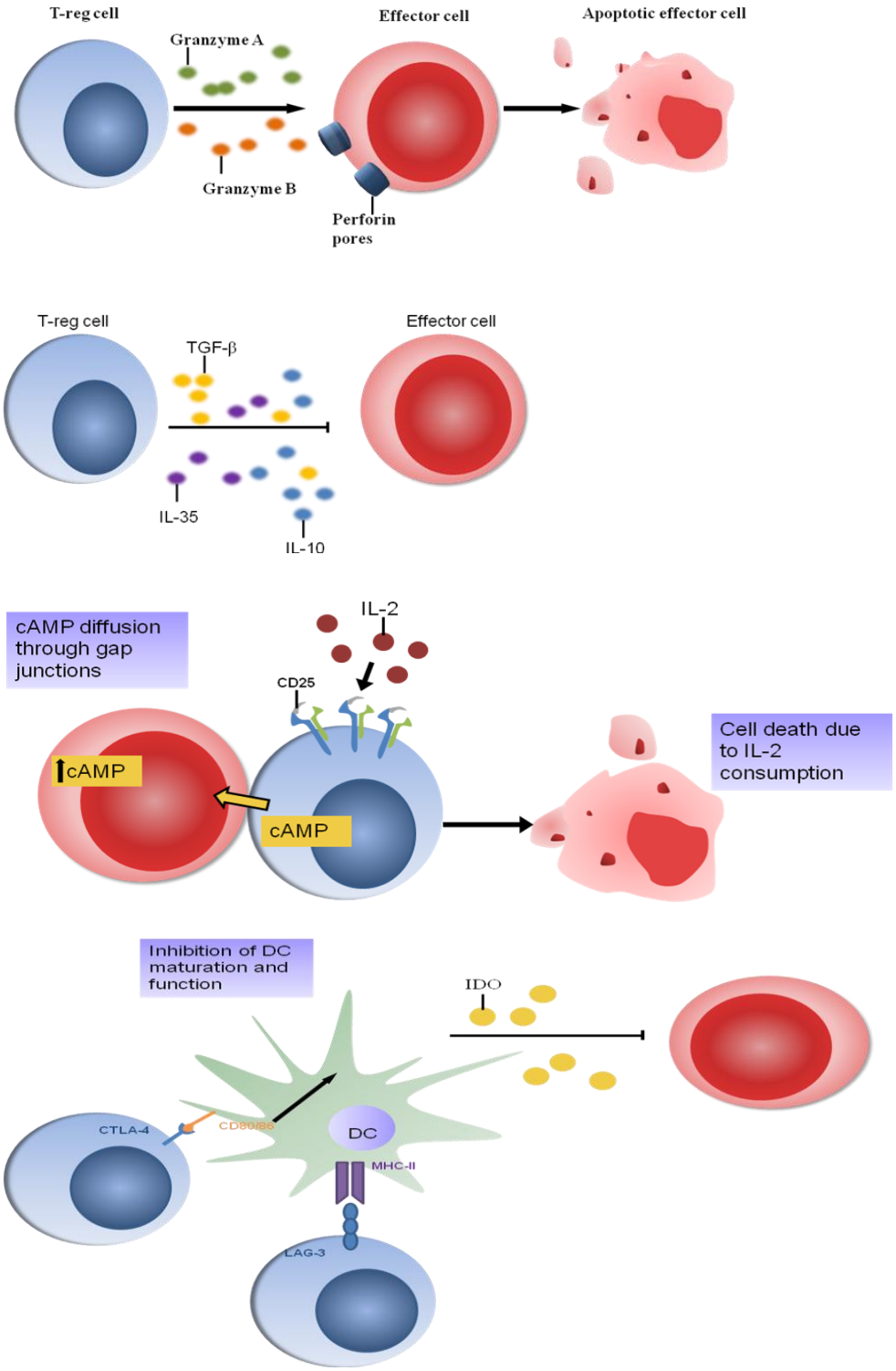


Figure 1.5 Mechanisms of suppression of natural T-regs.
Suppression of T effector cells by natural T-regs includes cell contact-independent mechanisms such as secretion of IL-10, TGF-β and IL-35 (a), granzyme A or B secretion (b) and increase of intracellular levels of cAMP (c). Cell contact-dependent mechanisms include inhibition of DC maturation and function through the down regulation of CD80, CD86 *via* CTLA4 function and MHC II molecules (adapted from Vignali et al. 2008).

1.3 IFN-γ and IL-10 in the immune system

1.3.1 IFN-γ; signalling pathways and functions

The study published by Wheelock in Science in 1965 reported that the exposure of human leukocytes to the mitogen phytohemagglutinin was able to induce the production of an interferon-like molecule with antiviral activity (Wheelock, 1965). The molecule Wheelock identified is today known as IFN-γ or type II interferon. The term "interferon" indicates the ability of this class of molecules to "interfere" with viral replications. Although this was the characteristic that allowed the identification of IFN-γ, it is now known that the structure and the receptors of IFN-γ share limited homology with the one of type I interferons (i.e. IFN-α and IFN-β) and its signalling on effector cells of the immune system, rather than its anti-viral function, mediates its predominant role (Pestka et al., 2004). The IFN-γ gene is located on the human chromosome 12 and encodes for a non-covalent homodimer (Zimonjic et al., 1995). Several transcriptional factors bind to the IFN-γ promoter positively regulating gene transcription suggesting that the activation of this gene is multifactorial and requires several steps. The promoter of IFN-γ contains binding sites for the transcription factors ying yang 1, nuclear factor of activated T cells (NFAT), activated protein 1, nuclear factor kB and the master regulator of T_H1 differentiation, T-bet (Kiani et al., 2001; Sica et al., 1997; Sweetser et al., 1998; Szabo et al., 2000). Hence, the activation of IFN-γ is dependent on and can be initiated by different stimuli. The receptor for IFN-γ belongs to the type II cytokine receptor class and comprises of two subunits known as IFNGR1 and IFNGR2, which upon interaction with IFN-γ oligomerize and form a heterodimer (Ahmed and Johnson, 2006). The IFN-γ receptor signalling chains have binding motifs for the Janus tyrosine kinase (Jak)1, Jak2 and STAT1 which are recruited during signal transduction and mediate the intracellular pathways of IFN-γ signalling (Katsoulidis et al., 2005). Upon the interaction between IFN-γ and its receptor, Jak2 auto-phosphorylates and consequently induces Jak1 phosphorylation. In turn, Jak1 phosphorylates target tyrosines on the IFN-γ receptor allowing the docking and formation of STAT1 dimers (Leonard, 2001). Activated STAT1 dimers dissociate from the receptor intracellular chains and translocate to the nucleus where they promote or inhibit transcription of target genes (Ehret et al., 2001; Nguyen et al., 2000). IFN-γ is a major effector cytokine of T_H1 cells as it promotes the development of T_H1 responses (see paragraph 1.1.7) both on a transcriptional level (e.g. positive

regulation of T-bet and inhibition of GATA-3) and on a cellular level (e.g. induction of T_H1 skewing cytokines by APCs). Several studies have shown that IFN-γ is not only the main T_H1 cytokine but that it is also produced by other immune cells including CTLs, NK cells, NKT cells, monocyte/macrophage and DCs (Mosmann et al., 1997; Leite-De-Moranes et al., 1998; Wherry et al., 1991). Whilst innate production of IFN-γ is thought to be associated with promotion of inflammation during the early phases of an immune response towards a pathogen, the production of IFN-γ by T cells is known to be important in orchestrating adaptive immune responses (Schoenborn and Wilson, 2007). The production of IFN-γ is mainly induced in response to IL-12 or IL-18 cytokines, which can induce an increase of IFN-γ production by macrophages, NK cells and T cells (Akira, 2000; Munder et al., 1998; Schindler et al., 2001). Other activating signals can induce IFN-γ production independently of cytokine signalling; for example NK cells, via the engagement of TLRs and NKG2D, can secrete IFN-γ (Girart et al., 2007). Macrophage infection or activation up-regulates the production of IL-12 and chemokines such as macrophage inflammatory proteins (MIPs). Such chemokines are pivotal in promoting migration of innate cells, such as NK cells, to the site of infection inducing the concomitant production of IL-12, which in turn promotes the synthesis and secretion of further IFN-γ (Salazar-Mather et al., 2000). Numerous IFN-γ regulated proteins involved in the migration of immune cells to the site of inflammation have been described. IFN-γ has been shown to up-regulate the expression of chemokines such as chemokine (C-C motif) ligand 5, MIP-1α and IFN-inducible protein 10; these factors promote leukocyte diapedesis and the induction of adhesion molecules expression such as intercellular adhesion molecule 1 (ICAM) and vascular cell adhesion molecule-1 (VCAM-1) (Gil et al., 2001; Jesse et al., 1998; Taub et al., 1993a; Taub et al., 1993b).

In addition to regulating cell trafficking and cytokine production, IFN-γ impacts several other pathways of cell mediated immunity. These include: MHC class I and class II antigen presentation pathways, macrophage activation, B cell isotype switching and T helper differentiation (Schoenborn and Wilson, 2007). IFN-γ stimulation induces the up-regulation of both the class I MHC heavy chain and the β_2-microglobulin which interacts with the class I MHC heavy chain for the formation of the MHC I. This associates with foreign antigens favouring presentation to CTLs in order to promote cytotoxic activity mainly against viral antigens (Decker et al., 2002; Johnson and Pober, 1990; Wallach et al., 1982). IFN-γ also impacts on the

composition of the proteasome subunits favouring the formation of the immunosome; this is achieved by up-regulating the expression of MHC encoded subunits (i.e. Low Molecular Mass Polypeptide 2 and 7) which can replace the β_1 , β_2 and β_5 subunits of the proteasome increasing the quantity and efficiency of MHC I peptide generation and loading (Belich et al., 1994). IFN-γ has also a key role in the up-regulation of MHC II molecules mainly in APCs including B cells, DCs, monocytes and macrophages (Cresswell, 1994). IFN-γ up-regulates MHC II expression by increasing the expression of the invariant chain (I_i), and of cathepsins B, H and L, which are cysteine proteases implicated in the processing of antigens and in the maturation of MHC II molecules at the level of lysosomes (Lafuse et al., 1995; Lah et al., 1995). The key mediator regulating the expression of such genes is the class II transactivator (CIITA), an IFN-γ induced protein which acts as the limiting component in a multimeric complex involved in the transcriptional regulation of MHC II associated genes (Chang and Flavell, 1995).

One of the most important functions of IFN-γ is the activation of macrophages characterised by enhanced phagocytic and microbicidal functions in the contest of intracellular microbes infections. Macrophages stimulated by IFN-γ in conjunction with other activating signals, such as TLRs engagement by microbial antigens or CD40 signalling, up-regulate the expression phagolysosomal enzymes (Ahmed and Johnson, 2006). A pivotal enzyme in the activation of macrophages is the inducible nitric oxide synthetase (iNOS) (Ma et al., 2003). iNOS catalyses redox reactions producing nitric oxide a key mediator of microbe killing by macrophages (Shiloh et al., 1999). The microbicidal activity of macrophages is also enhanced by the IFN-γ mediated up-regulation of enzymatic subunits of the NADPH oxidase (namely $gp91^{phox}$ and $gp67^{phox}$). The NADPH oxidase generates reactive oxygen species such as superoxide O^{2-} (Cassatella et al., 1990). The importance of the NADPH oxidase activity is highlighted by human genetic deficiencies which cause chronic granulomatous diseases with recurrent and fatal infections (Song et al., 2011). IFN-γ has been also implicated in the regulation of B cell isotype switching. Different cytokines are involved in such process with IL-4 playing a preponderant role (Pene et al., 1988). However, it has been reported that IFN-γ stimulates B cell isotype switching favouring the subclasses IgG2a and IgG2c which are also involved in classical complement activation (Bossie and Vitetta, 1991; Finkelman et al., 1988; Yoshida et al., 1994). Similarly to the opposing effects of IFN-γ to IL-4 on T helper skewing (i.e.

favouring of T_H1 rather than T_H2 differentiation) IFN-γ has been found to have an antagonistic effect to the IL-4 mediated isotype switching by inhibiting isotypes such as IgE and IgG1 (Coffman and Carty, 1986).

1.3.2 IL-10; signalling pathways and functions

The immunosuppressive properties of IL-10 were clear since its discovery as it was initially identified as a cytokine synthesis inhibitory factor of T_H1 induced cytokines such as IFN-γ, TNF and IL-2 (Fiorentino et al., 1989). IL-10 is now widely recognised as the canonical immunosuppressive cytokine and numerous studies have shed light on its structure and signalling pathways highlighting its therapeutic potential in inflammatory, infectious and autoimmune pathologies (Moore et al., 2001). The IL-10 gene is located on human chromosome 1 and encodes for a heterodimer of 36 kDa. The two subunits of IL-10 consist of six amphipathic helices and are stabilized by intra-molecular disulphide bridges (Zdanov et al., 1995). The regulation of the IL-10 gene is complex and several transcription factors have been described. STAT3, the transcription factor specific protein 1 (SP1) and the IFN-regulatory factor 1 (IRF1) are among the main trans-activators of the IL-10 promoter described for human and mouse macrophages and T cells (Asadullah et al., 2003; Staples et al., 2007; Ziegler-Heitbrock et al., 2003). The IL-10 promoter comprises also binding sites for GATA3, which was shown to be a key transcription factor for IL-10 in T_H2 cells, and c-Maf, which enhances IL-10 expression in LPS stimulated macrophages and has been associated to IL-10 production in different T helper subsets (Cao et al., 2005; Shoemaker et al., 2006). The receptor for IL-10 belongs to the type II cytokine receptor family and is composed of two chains, known as IL-10R1 and IL-10R2 characterised by extracellular, transmembrane and intracellular domains (Liu et al., 1994). Jak1 and the Tyrosine Kinase-2 (Tyk2) constitutively interact with the IL-10R1 and IL-10R2 respectively, mediating the first signalling events upon IL-10/IL-10R interaction (Finbloom and Winestock, 1995). Upon activation Jak1 and Tyk2 phosphorylate tyrosine residues of the IL-10R1 intracellular chain promoting the association of STAT3 to the IL-10R1 (Donnelly et al., 1999). Phosphorylation of STAT3, STAT1 and STAT5 proteins allows the generation of homo- or hetero-dimers which translocate into the nucleus where they bind to STAT-binding elements and regulate the transcription of several genes (Schaefer et al., 2009; Tsuji-Takayama et

al., 2008). STAT3 has been implicated in the regulation of the transcription of anti-apoptotic and cell-cycle progression genes such as BCLXL, cyclins and c-Myc (Otero et al., 2006; Pandey et al., 2009). Important mediators of IL-10 signalling are the suppressors of cytokine synthesis (SOCS)-1 and SOCS-3 which have been shown to be induced by IL-10 signalling via a Jak-STAT dependent pathway (Yoshimura et al., 2007). In particular, SOCS-1 inhibits IFN-γ and IL-4 signal transduction by binding to Jak kinases and interfering with their enzymatic activity (Alexander et al., 1999). SOCS3 has been shown to inhibit the expression of TNF and IL-1 in monocytes (Meisel et al., 1996). Importantly IL-10 has also been shown to negatively regulate the p38/MAPK activation pathway consequently inhibiting TNF translation by interfering with the LPS-induced polysome coupling of TNF-mRNA in macrophages (Kontoyiannis et al., 2001).

IL-10 can be produced by many cell types of the innate and adaptive immune system; in the latter IL-10 production is not only associated to T cells with regulatory activity (paragraph 1.2); T helper cells subsets and $CD8^+$ T cells are able to produce IL-10 (Moore et al., 2001). Furthermore, IL-10 production by innate cells of the immune system has been reported. In fact, macrophages, DCs and neutrophils can express IL-10 in response to appropriate stimuli. IL-10 production by macrophages and myeloid DCs can be stimulated upon the engagement of TLR2, TLR4 and TLR9 (Boonstra, A. et al., 2007; Siewe et al., 2006). TLR-independent production of IL-10 has been demonstrated as in the case of dectin 1 (a C-type lectin) stimulation of DCs (Rogers, et al., 2005). Immunosuppressive agents (e.g. Vitamin D3) and IL-10 itself have been shown to induce the differentiation of a specific subset of DCs known as tolerogenic DCs due to their ability to induce T cells with regulatory activity *in vitro* and *in vivo* (Adorini et al., 2004; Wakkach et al., 2003; Chu et al., 2012). Importantly other innate immune cells associated mainly with pro-inflammatory functions have been shown to produce IL-10 upon TLR and/or C-type lectin receptors engagement. Neutrophils (Zang et al., 2009) and mast cells (Grimbaldeston et al., 2007) can in fact be sources of IL-10 upon such stimuli. Moreover, in the adaptive immune system T cells do not represent the only source of IL-10. B cells with IL-10-mediated regulatory activity have been identified in mouse and human systems and their impaired function has been described in settings of chronic inflammation and autoimmunity (Blair et al., 2010; Burdin et al., 1997). The functions exerted by IL-10 are numerous and go beyond the initially identified suppression of IFN-γ, IL-2 and TNF. Acting on T helper subsets IL-

10 negatively affects proliferation and the activities of several cytokines including IL-2 and IL-4 consequently inhibiting T cell differentiation and function (Petska et al., 2004). IL-10 inhibits the expression of MHC class II and costimulatory molecules such as CD80 and CD86 on monocytes, macrophages and DCs and also inhibits IL-12 production (Aste-Amezagaet al., 1998; Ding et al., 1993). IL-10 has also been shown to negatively affect chemokine production in DCs, interfering and blocking their migration to lymph nodes (Demangel et al., 2002). In particular, IL-10 seems to down-regulate the expression of CCR7 and up-regulate the expression of CCR5 impacting on DCs chemotaxis *in vitro* and impairing the *in vivo* homing of DCs to secondary lymph nodes (Takayam et al., 2001). Adhesion molecules are also negatively regulated in human monocytes by IL-10; in these cells IL-10 has been shown to down-regulate the expression of intercellular adhesion molecule 1 (ICAM-1) and of B7 molecules (Willems et al., 1994). Moreover, IL-10 promotes the development of B1 cells (Ishida et al., 1992) and in combination with IL-18, favours the cytotoxic activity NK cells (Cai et al., 1999). The importance of IL-10 in the maintenance of immune homeostasis has been reported *in vivo* by human mutations of the IL-10 receptor chains which are associated to colitis and by murine models of IL-10 deficiency characterised by fatal immunopathology in several infection models including Toxoplasma gondii, malaria, and Trypanosoma cruzi (Franke et al., 2008; Taub et al., 1993a) Gazzinelli et al., 1996; Hunteret al., 1997; Li et al., 1999).

1.4 The complement system – an outline

The canonical view of complement places this enzymatic cascade as an effector mechanism of the mammalian innate immune system. Since its discovery the main effector functions of the complement system have been related to bacterial lysis, opsonisation and phagocytosis of invading microbes. However, the understanding of complement as a sole innate effector system able to rapidly react to pathogen associated molecular patterns (PAMPs) has been challenged in the past decades. New roles have emerged for complement in tissue homeostasis and adaptive immunity and multiple efforts from the scientific community have shown how complement guides multiple other processes that include: initiation of local inflammation, clearance of

immune complexes, processing of apoptotic and necrotic cells, shaping and regulation of the humoral and cell-mediated adaptive immune responses (Ricklin et al., 2010).

The link between complement and adaptive immunity has been evident since the discovery of the complement activity against bacteria by Jules Bordet in 1901. In fact, the term complement was used by Bordet to explain how a heat sensible fraction present in immunized serum was essential to integrate (i.e. complement) the activity of antibodies for the lysis of cholera vibrios (Bordet et al., 1901). This was the first observation indicating not only an important effector function of the complement system but also suggesting a modality of humoral immunity regulation by complement components. Several mechanisms of adaptive immune responses regulated by complement have been highlighted and are described in the following paragraphs.

1.4.1 Complement activation and key effector functions

To date the components of the complement system account for more than 30 players including proteases, anaphylatoxins, receptors and regulators (Carroll, 2008). Three different activation pathways of the complement system are widely accepted and converge into a key process; the cleavage of the third component of the cascade C3 into C3a and C3b (Figure 1.6). The antibody dependent pathway of complement activation inferred by Bordet is known as classical pathway (CP) (Lafuse et al., 1995). The activation of the complement cascade in the CP pathway requires the binding of complement component C1 to the Fc region of IgG clusters or IgM antibodies that have recognised antigen (Figure 1.6). Thanks to the C1-associated proteases C4 is cleaved into C4a and C4b. C4b binds C2 which is also cleaved into C2a and C2b to generate the CP C3 convertase C4b2b (a change in the nomenclature identifies C2b as the protease fragment rather than the small non proteolytic fragment)(Ricklin et al., 2010).

The alternative pathway (AP) refers to the constant hydrolytic ‘tick over’ of C3, a process that is innocuous to the host (Lafuse et al., 1995). The hydrolysis of C3 generates the C3(H_2O) accessible to the binding of factor B which in turn is cleaved by factor D generating a soluble C3 convertase, C3(H_2O)Bb which can cleave further C3;

the generated C3b can then bind on target surfaces covalently *via* an activated thioester and initiate the generation, in the presence of factor B and factor D, of the AP C3bBb convertases. Properdin is also able to induce the alternative pathway activation; properdin is a PRR able to recognise foreign cells and to bind fluid phase C3b in order to generate the C3bBbproperdin convertase (Spitzer et al., 2007).

The lectin pathway is initiated by the recognition of carbohydrates structures expressed on pathogens by different lectins including the mannan binding lectin (Hambleton et al.) and the ficolins (Carroll, 2004; Gros et al., 2008). The details of each activation pathway can be found in Figure 1.6. Briefly, MBL interacts with an MBL associated serine protease (MASP) which cleaves C4 and/or C2 to generate the C3 convertase C4b2b. The lectin pathway is likely to be the oldest in terms of evolution since prochordates (Le Friec and Kemper, 2009) present MBL like proteins. The presence of complement components in different evolutionary phyla is a reflection of the efficacy and the importance of such system as a defence mechanism. The activation of the complement pathway on a target cell induces generation of further C3b deposition and opsonisation or bind to C3 convertases generating the C5 convertases (AP, C3bBb3b or LP and CP, C4b2b3b) (Ricklin et al., 2010). The C5 convertases cleave C5 in C5a and C5b. In the terminal pathway of the complement activation C5b interacts with C6 and C7 and once it is inserted in the membrane it interacts with C8. Only when C9 is bound complement membrane attack complex (MAC) activity is maximal and lytic pores can be formed to promote lysis of pathogens such as gram negative bacteria and parasites (Bhakdi and Tranum-Jensen, 1988).

The three complement activation pathways generate C3 and C5 fragments of circa 9 kDa known as anaphylatoxins C3a and C5a (Carroll, 2004). These are potent signal triggers for the G protein-coupled receptors; C3aR and C5aR. Such receptors are expressed on mast cells, eosinophils, basophils, neutrophils and lymphocytes; they participate to inflammatory processes by inducing the recruitment and activation of the above mentioned cell types and by promoting macrophage phagocytosis (Gerard and Gerard, 1991; Itoh et al., 1991). Parallel to the effect of the anaphylatoxins is the activity of opsonins among which C3b, C4b and downstream product C3d are key in inducing phagocytic activity in neutrophils and macrophages *via* the binding of the

respective complement receptors (i.e. CR1 and CR2) (Dempsey et al., 1996; Law et al., 1980; Ricklin et al., 2010).

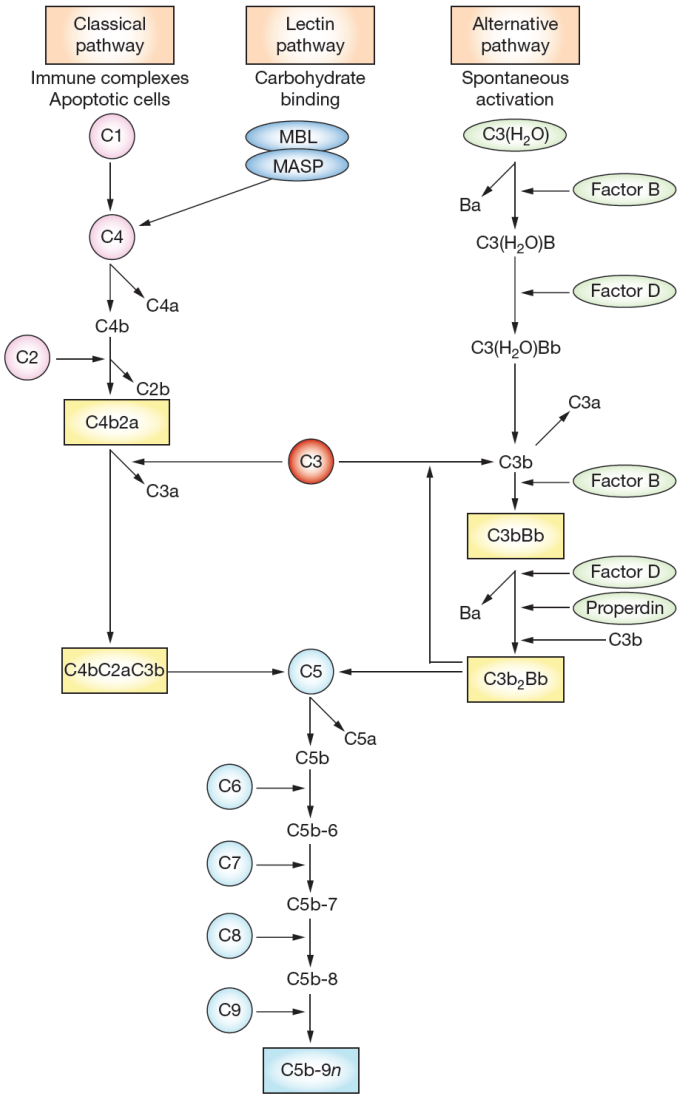


Figure 1.6 Complement cascade.
The activation of the complement cascade *via* the classical pathway requires the binding of complement multimeric complex of C1 to the Fc region of IgG clusters or IgM antibodies that have recognised antigen. C1 is composed of C1q –the molecule recognising the Fc region-, $C1r_2$ and $C1s_2$, two serine proteases. By binding two Fc regions of immunoglobulins C1q allows for the associated protease C1r to cleave and activate C1s. As a consequence of C1s activation, C4 is cleaved and C4b binds C2 which is then cleaved into C2a and C2b. This generates the C3 convertase C4b2b. The lectin pathway is initiated by mannose-binding lectins (Hambleton et al.) or ficolins recognised sugars. MBL associated serine proteases (MASPs) cleave C4 and/or C2 and generate the C3 convertase C4b2b. The AP is initiated by the spontaneous hydrolysis of C3 to $C3(H_2O)$ which can bind factor B (fB). Bound fB can be cleaved by factor D allowing the generation of the AP C3 convertase C3bBb. Amplification of the complement cascade by C3 convertases favours binding of C3b to either CP, AP or

lectin pathway C3 convertases generating C5 convertases which cleave C5 into C5a and C5b. The association of the following fragments of the complement cascade (i.e. C6-9) to C5b determines the formation of the membrane attack complex and promoting cellular lysis (Lafuse et al., 1995).

1.4.2 Complement regulation

The autocatalytic nature of the complement amplification and the indiscriminate diffusion of complement effector molecules to surrounding surfaces require a rapid and tight control of the cascade's activation to prevent unwanted deposition on self (Kohl, 2006). This need is reflected in the fact that the number of complement regulators and inhibitors is actually higher than the core zymogens identified in the complement system (C1 to C9) (Table 1.1). Complement regulators/inhibitors are divided in fluid phase or membrane bound; they are essential in allowing the enzymatic cascade to neatly differentiate between the host and non-self surfaces or self-modified cells (Carroll, 2004). This allows avoiding acute and/or chronic activation that would otherwise produce excessive damage among the tissues surrounding the site of infection (Zipfel and Skerka, 2009). Fluid phase complement regulators are present in the blood, lymph, synovial and vitreous fluids. They include alternative pathway inhibitors such as Factor H, FHL1 and properdin and classical and lectin pathway inhibitors such as C1 inhibitor and C4 binding protein (C4BP) (Table 1.1). Membrane bound regulators include CD35 (CR1), CD46, CD55 (DAF), CD59 and CRIg (complement receptor of the immunoglobulin family) and are more generally directed towards C3 and C4 inhibition rather than being specific for a particular pathway as the fluid phase inhibitors.

Complement inhibitors can: 1. Bind directly to their target (C1 inhibitor) and inhibit its function, 2. Act as cofactors for the factor I-mediated cleavage of C3b and C4b (cofactor activity; factor H, C4BP, CD46 –i.e. membrane cofactor protein, MCP- and CD35 –i.e. complement receptor 1, CR1-) 3. Accelerate the decay of convertases (CD35 and CD55 –decay accelerating factor, DAF-) or 4. Prevent the formation of the MAC and the terminal pathway formation (CD59, clusterin and vitronectin). Further additional proteases such as carboxypeptidase N can degrade the anaphylatoxins C3a and C5a respectively into C3a desArg and C5a desArg which have less potent pro-inflammatory properties.

Regulator	Alternative name	Point of Action	Ligand	Cell surface binding or expression	Function
Soluble regulators and effectors					
Factor H	None	AP	C3b and C3d	Acquired to surface	Cofactor of Factor I and acceleration of AP C3 convertase decay
FHL1	None	AP	C3b	Acquired to surface	Cofactor of Factor I and acceleration of AP C3 convertase decay
Properdin	None	AP	C3	Binds to apoptotic surfaces	Stabilization of AP convertases
Carboxy-peptidase N	Anaphylatoxin inactivator	CP and LP	C3a, C4a and C5a	NA	Inactivation of anaphylatoxins C3a and C5a
C4BP	None	CP and LP	C4	Acquired to surface	Cofactor for factor I and acceleration of CP C3 convertase decay
C1q	None	CP	IgG and IgM IC	Binds to apoptotic surfaces	Activation of CP
C1INH	None	CP and LP	C1r, C1s and MASP2	NA	Blocks serine protease, suicide substrate for C1r, C1s, MASP2, coagulation factors and C3b
CFHR1	None	TP	C5 convertase and TCC	Acquired to surface	Inhibition of C5 convertase and TCC assembly
Clusterin	SP-40, 40 and apolipoprotein j	TP	C7, 8B, C9 and TCC	NA	Transport of cholesterol, HDL, APOA1 and lipids
Vibronectin	S-protein	TP	C5b-7 and TCC	NA	Adhesion protein, fibronectin-mediated cell attachment and Arg-Gly-Asp site coagulation I immune defence against Streptococcus spp.
Surface bound regulators and effectors					
CR1	CD35, Immune adherence receptor	C3	C3b, iC3b, C4b and C1q	Nucleated cells, erythrocytes, B cells, leukocytes, monocytes and FDCs	Clearance of IC
CR2	CD21 and Epstein-Barr virus receptor	C3	C3dg, C3d and iC3b	B cells, T cells and FDCs	Regulation of B cell function, B cell co-receptor and receptor of C3d tagged ICs
CR3	MAC1,CD11b-CD18 and αMβ2 integrin	C3	iC3b and Factor H	Monocytes, macrophages, neutrophils, NK cells, eosynophils, myeloid cells, FDCs, $CD4^+$ and $CD8^+$ T cells	iC3b enhances the contact of opsonized targets, resulting in phagocytosis and adhesion by CR3
CR4	CD11c-CD18 and αXβ2 integrin	C3	iC3b	Monocytes and macrophages	iC3b-mediated phagocytosis
CRIg	VSIG4	C3	C3b, iC3b and C3c	Macrophages	iC3b-mediated phagocytosis and AP activation

Regulator	Alternative name	Point of Action	Ligand	Cell surface binding or expression	Function
Surface bound regulators and effectors					
CD46	MCP	C3	C3b and C4b	All cells except erythrocytes	C3 degradation, cofactor for factor I and factor H, and effector for T cell maturation
CD55	DAF	C3	C4b2b ad C3bBb	GPI anchor expression by most cell types including erythrocytes, epithelial and endothelial cells	Inhibition of TCC assembly
CD59	Protectin	TCC	C8 and TCC	GPI anchor expression by most cell types and most nucleated cells	Inhibition of TCC
C3aR	None	C3	C3a	Neutrophils, monocytes, eosinophils, APCs, T cells, astrocytes, neurons and glial cells	Immune cell recruitment and inflammation
C5aR	CD88	C5	C5a	Myeloid cells, neutrophils, monocytes, eosinophils, APCs, T cells endothelial cells and renal tubular cells	Immune cell recruitment and inflammation
C5L2	None	C5	C5a	Macrophages and neutrophils	Immune cell recruitment and inflammation and possibly acts as a decoy receptor
C1qR	CD93	CP	C1q	Monocytes and B cells	Phagocytosis and cell adhesion
SIGNR1	CD209	CP	C1q	DCs and microglial cells	Signalling and phagocytosis

Table 1.1 Description of complement regulatory and complement receptor proteins substrates and functions. Complement regulators can be divided in fluid phase and membrane bound. Fluid phase regulators include; Factor H, FHL1, properdin (AP inhibitors), C1 inhibitor, C4 binding protein (C4BP), CFHR1 and carboxypeptidase N (CP and lectin pathways inhibitors). Membrane bound regulators include CD35 (CR1), CD46, CD55 (DAF), CD59 and CRIg (complement receptor of the immunoglobulin family). Complement receptors (CR) CR1, CR2, CR3 and CR4 are expressed on different subsets of immune cells and are involved in multiples cellular processes (Zipfel and Skerka, 2009). APOA1, apolipoprotein A; C, complement component; C1INH, C1 inhibitor; C52L C5a receptor-like 2; CFHR1, complement Factor H related protein 1; DAF, decay-accelerating factor; DCs, dendritic cells; FDCs, follicular dendritic cells; FHL1, Factor H-like protein 1; GPI, glycosylphosphatidylinositl; HDL, high-density lipoprotein; ICs, immune complexes MASP2, mannan-binding lectin serine protease 2; MCP, membrane cofactor protein; NA, not applicable; SIGNR1, mouse homologue of DC-SIGN, SIGN-related 1; TCC, terminal complement complex.

1.4.3 Complement and adaptive immunity - B cells

Experimental evidence linking the complement system to humoral immunity has only become available several decades after the discovery of complement by Bordet in 1901. In the 1970s, following the identification of receptors for C3 on B cells (Nussenzweig, 1971), Pepys demonstrated that transiently depleting serum of C3 through cobra venom factor (which leads to consumption of circulating C3) impairs the humoral response towards T cell dependent antigens (Pepys, 1972). Moreover, mice deficient for complement fragments C1q, C3 and C4 (Cutler et al., 1998; Moon et al., 1997) and complement receptors CR1 (CD35) and CR2 (CD21) (Ahearn et al., 1996; Karp et al., 1996; Ochsenbein et al., 1999) do not develop a normal humoral response and are characterised by impaired antibody class isotype switching and a reduced size and number of germinal centres. Complement fragments also influence B cell activation and differentiation. The co-ligation of the BCR with the co-receptor CD21/19/81 through C3d coated antigens, lowers the threshold of B cell activation up to 10.000 fold (Dempsey et al., 1996). Further, survival signals mediated by the CD19/21/81 and the BCR have been shown to be vital for B cells in germinal centres. In fact, $CR2^{-/-}$ B cells are outgrown by WT CR2 B cells in an active humoral response; hence CR2 signals are fundamental in lowering BCR reactivity which allows for B cell maturation (Barrington et al., 2005). Although most of the *in vivo* evidence described so far comes from mouse models, also human genetic deficiencies stress the importance of complement in B cell responses. C3 and C4 deficiencies are associated to impaired antibody responses towards T cell dependent and independent antigens (Carroll, 2008). In particular, Drouet's group recently showed that a C3-deficient paediatric patient was unable to generate B cell memory; in fact attempt of vaccination did not translate in long-term antibody responses (Ghannam et al., 2008).

1.4.4 Complement and adaptive immunity - T cells

Given the strong links between complement and the activation/maturation of B cells, a role for the complement system in the shaping of T cell responses has been easily envisaged. Most of the information available to date on the complement/T cell axis has been generated using small animal models (Botto et al., 2009; Zhou, 2012). The most studied complement molecules impacting on T cell immunity are the anaphylatoxins receptors and complement regulators (e.g. CD46, CD55 and CD59) (Kemper et al., 2007)**.** Because CD46 is only expressed widely in humans but not on rodent somatic cells (Riley-Vargas et al., 2004), a clear separation between data generated using mouse models and human cell lines/primary cells is necessary. Thus, whilst the following background information is in large part derived from mouse models, human related studies will be highlighted to clarify what is known in the two systems. Given that the focus of the study in this thesis has been on T cells, the following paragraphs will describe the indirect and direct effect of complement on T cell immunity.

1.4.5 Complement - indirect effects on the shaping of T cell responses

The influence of complement on T cell biology was initially identified to be mainly *via* the modulation of the function of APCs. As described above (see paragraph 1.3), target opsonisation is one of the key functions of complement; indeed APCs derived from $C1q^{-/-}$, $C3^{-/-}$, factor $B^{-/-}$ or factor $D^{-/-}$ mice have defective antigen uptake/processing and lead to weaker allospecific T cell reactivity (Heeger et al., 2005; Kerekes et al., 2001; Lalli et al., 2007; Lalli et al., 2009; Peng et al., 2008; Peng et al., 2006; Schoenberger et al., 1998; van Montfoort et al., 2007). Several studies have highlighted how local synthesis of complement components upon cognate interactions between APC and T cells is a key process in T cell immunity (Heeger et al., 2005; Lalli et al., 2007; Peng et al., 2008; Strainic et al., 2008). Heeger's study (2005) initially identified the importance of local complement production in mouse APCs. In this study, absence of DAF (CD55) on APCs induced a stronger T cell proliferation and cytokine production in comparison to WT APCs due to the increased production of local complement components. Further studies by Peng et al., (2006), Strainic et al., (2008) and Pavlov et al., (2008) supported the importance of immune cell-derived complement describing the impact of local synthesis of alternative pathway complement components such as C3, Factor B, Factor D and C5 on the way APCs shape T cell responses *in vitro* and in *in vivo* models of transplantation and infection. Several studies demonstrated that engagement of C3aR and C5aR G-protein-coupled receptors on human monocytes-derived DCs and on mouse DCs induce a down-regulation of cAMP levels and promotes activation of PI3k/AKT, ERK and NF-kB pathways (Peng et al., 2009; Schoenberger et al., 1998; Strainic et al., 2008). Importantly these signalling events promote the up-regulation of key APC co-stimulatory molecules including MHC II, CD80, CD86 and CD40L and the production of T_H1 and T_H17 skewing cytokines, mainly IL-12 and IL-23 in both human and mouse systems (Hawlisch et al., 2004; Schoenberger et al., 1998; Strainic et al., 2008). Interfering with either APC-T cell interaction at the level of B7-1/CD28 recognition or with C3aR and C5aR signalling, translates into lower expression of co-stimulatory molecules (namely MHC II, CD80, CD86), lower production of IL-12 and as a consequence weaker stimulation of T_H1 cells in comparison to WT APCs (Peng et al. 2006; Lalli et al., 2007; Strainic et al., 2008). Importantly anaphylatoxin signalling seems to be involved in T_H2 biology; in fact in a mouse model of asthma C3aR deficiency was found to be associated with

decreased IL-4 production and T_H2 responses with consequent protection against airway hyperreactivity (Drouin et al., 2002). Further examples of how complement can influence T cell function *via* DCs are given by CD46. Engagement of CD46 on human DCs promotes IL-23 production which induces higher secretion of IL-17 by $CD4^+$ T cells (Vaknin-Dembinsky et al., 2008). Pathogens binding to CD46 have also been shown to affect T cell responses. Neisseria species, human herpes virus 6 and measles virus bind to CD46 reducing the expression of IL-12 in APCs thus inhibiting T_H1 responses and favouring infection through cellular immune suppression (Karp and Wills-Karp, 2001). Interesting insights have risen regarding the network of TLR and complement signalling in APCs. Hawlisch et al., (2005) reported of the negative regulation of C5a on TLR4 and CD40 signalling in IFN-γ primed macrophages which consequently decreased cytokine production (IL-12, IL-23 and IL-27) and limited T_H1 development (Hawlisch et al., 2005). A more recent study from Kohel's group determined the importance of the C5aR expressed on splenic DCs regarding its influence in the differentiation of T-reg cells and T_H17. In the absence of C5a signalling on TLR2 stimulated splenic DCs there was a down modulation of T_H1 skewing cytokines and an increase in the production of T_H17 and T-reg skewing cytokines including TGF-β, IL-23 and IL-6 (Sweetser et al., 1998).

1.4.6 Complement; direct effects on the shaping of T cell responses

Pivotal complement mediated signals on T cells can also be directly delivered by the anaphylatoxins C3a and C5a (Zhou et al., 2011). The expression of the C3aR and C5aR has been documented in both mouse and human T cells. Whilst human C3aR is only expressed upon T cell activation (Werfel et al., 2000), the C5aR is constitutively expressed at low levels and upregulated following T cell activation (Nataf et al., 1999). Mouse T cells have been shown to upregulate the expression of C3aR and C5aR upon activation and to induce signalling through the PI3k and Akt pathways. Such signals transmit anti-apoptotic signals (through the up-regulation of B-cell lymphoma 2) and promote proliferation (Lalli et al., 2008; Strainic et al., 2008).

Several complement receptors and regulators can function as co-stimulators of T cell activation and influence T cell immunity (Kemper et al., 2007; Zhou, 2011). The expression of CR1 been shown to be present in only 15% of circulating human T cells, thus its general role in T cell regulation is unclear. However, functional studies with monoclonal antibodies towards CR1 have shown that engagement of this receptor

translates in an inhibition of T cell proliferation and of IL-2 and IFN-γ synthesis (Mazzucchelli et al., 1996).

T cell immunity has been shown to be also directly regulated by DAF (CD55) in a study by Liu et al. (2005). The authors demonstrated that in the absence of DAF, experienced T cells become "hyper-responsive" upon antigen exposure increasing IFN-γ and IL-2 production (whilst decreasing IL-10) in comparison to WT T cells (Cao et al., 2005). To attest for the importance of these findings in a pathological setting the authors investigated the effect of DAF deficiency on the animal model of multiple sclerosis known as EAE and showed that DAF deficiency correlated with the highest disease score. The hyper-reactivity of DAF-deficient T cells was due to the increased availability of complement fragments (i.e. C3a and C5a) due to a decrease in complement inhibition in DAF deficient cells. However, as described under 1.3.5 later studies showed that local complement fragments are essential in regulating both APCs and T cells functions, further extending the initial findings by Liu's. The role of DAF has also been investigated in the human immune system. The co-engagement of CD3 and DAF (CD55) on $CD4^+$ T cells in the presence of IL-2 is able to induce lymphocyte proliferation and up-regulation of the activation markers CD25 and CD69. T cells activated through CD3 and DAF, *via* monoclonal antibodies or natural ligands (i.e. CD97) also showed a significant increase in the secretion of IL-10 and GM-CSF. Importantly the authors showed that the effects mediated by CD3 and DAF were not due to the decreased complement inhibition but rather to DAF signalling (Capasso et al., 2006). Similarly, Korty et al., (1991) showed that the engagement of the MAC inhibitor CD59, in the presence of phorbol myristate acetate (PMA) and ionomicin and of anti-Ig antibodies, was also able to induce signalling events in human resting T cells. The authors described an increase of intra-cytoplasmic Ca^{2+} and IP_3 and a concurrent increase in IL-2 production and T cells proliferation (Korty et al., 1991). Further studies on CD59 on human $CD4^+$ T cells have shown that CD59 expression is increased in the memory subset of T cells. Blocking CD59 on T cells stimulated through CD3 in the presence of APCs translated in a dose dependent increase of T cell proliferation and IFN-γ production in comparison to CD3 only stimulated T cells (Sivasankar et al., 2009). A role of complement regulator CD46 in T cell regulation has also been described (Kemper et al., 2003.). Given the central role of CD46-mediated signalling events on human T cells in this thesis, the role of CD46 will be discussed in a separate paragraph (see paragraph 1.4).

1.5 CD46

1.5.1 CD46; structure and signaling

CD46 is a type I transmembrane glycoprotein firstly identified as a complement inhibitor but involved in many biological processes including pathogen infection (Gaggar et al., 2003), fertilization (Riley et al., 2002) and T cell regulation (Shalapour et al.). In humans, the gene encoding CD46 is located on chromosome 1q32 within the regulators of complement activation gene cluster (Lublin et al., 1988). CD46 comprises four short consensus repeats (SCRs), an alternatively spliced region (B and/or C regions), highly glycosylated and rich in the aminoacids serine, threonine and proline (STP) followed by 12 amino acids and a transmembrane region (Figure 1.7). Due to alternative splicing, CD46 can be expressed with two distinct cytoplasmic tails: CYT-1 (16 amino acids) or CYT-2 (23 amino acids), both containing signaling motifs and putative nuclear localization sequences. The co-expression of the two isoforms on all human nucleated cells leaves their specific functions difficult to analyze. An important limitation in the analysis of CD46 signaling is the lack of a small animal model. Indeed, rodents only express CD46 on the spermatozoal acrosome membrane, and CRRY, the closest functional homologue of CD46, only shares the complement inhibitor features but not the T cell modulatory or pathogen-binding functions (Fernandez-Centeno et al., 2000; Foley et al., 1993).

1.5.2 CD46 functions

1.5.2.1 Complement regulation

The complement regulatory function of CD46 was the first to be described (Lublin et al., 1988). CD46 acts as a cofactor for the factor-I mediated cleavage of C3b and C4b deposited on host tissue, and therefore plays a key role in the inhibition of unwanted complement activation on self-tissue (Kemper et al., 2005**).** This complement regulatory function has been attributed to SCRs 2-4 through which CD46 binds C3b or C4b (Persson et al., 2010). Mutations that occur in the CD46 gene have been shown to predispose to the complement mediated disease (Richards et al., 2003), atypical Hemolytic Uremic Syndrome. HUS is characterized by microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure that occur as a consequence of microthrombi in the glomeruli (Maga et al., 2011). Different mutations of the *CD46* gene can affect either the availability of CD46 to bind C3b or the expression levels of CD46 on the endothelium. As a consequence the impaired ability to inhibit C3b deposition on host cells determines an increased activity of complement activation on the host through alternative pathway activation and increased deposition of C3b and thus damage of the endothelium (Goodship et al., 2004).

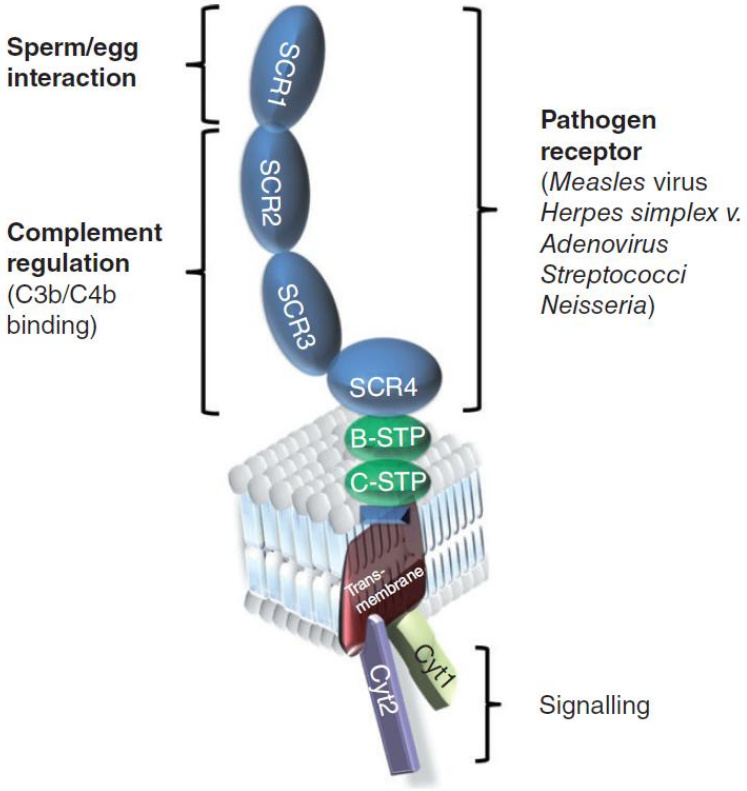


Figure 1.7 CD46 Structure.
The extracellular portion of CD46 comprises four short consensus repeats (SCR1 to 4) which are exploited by pathogens as docking sites for cellular infection. SCR1 is responsible for the sperm-egg interaction whilst SCR2 to 4 are involved in the complement regulatory function of CD46. Recent insights on the structure of the extracellular portion of CD46 by Personn et al., (2010) suggest a "bend" between SCR3 and SCR4 schematically represented in the figure. The highly glycosylated and serine, threonine and proline (STP)-rich regions B and/or C are generated by alternative splicing and are followed by a 12 amino acids peptide linked to the transmembrane region. Each of the two distinct cytoplasmic tails, CYT-1(16 amino acids) or CYT-2 (23 amino acids), contains phosphorylation sites (e.g. for protein kinase C in CYT1 and for Src in CYT2) and mediates intracellular signaling (Cardone et al., 2011).

1.5.2.2 Pathogen receptor

CD46 is also an entry receptor for several pathogens. Both measles virus (MV) and adenoviruses bind the extracellular domains SCR1 and 2 of CD46, *via* the viral proteins hemaglutinin and fiber knob, respectively (Gaggar et al., 2003; Naniche et al., 1993). The interaction of human herpesvirus-6 (HHV-6) and CD46 requires SCR2 and SCR3 (Santoro et al., 1999). Engagement of CD46 by pathogens has a direct impact on

the innate response by modulating cytokine secretion of innate immune cells and attests to its ability to transmit intracellular signals. For instance, MV and HHV-6 inhibit respectively primary monocytes and macrophages production of IL-12, a key cytokine involved in the T_H1 differentiation and activation of both NK and T cells (Smith et al., 2003). Moreover, recombinant adenovirus 35 is able to inhibit DCs maturation and naïve $CD4^+$ T cell proliferation and IL-2 production (Adams et al., 2011). *Streptococcus pyogenes* interacts with SCR3 and SCR4 of CD46 through the bacterial M protein and is able to induce IL-10 production in activated $CD4^+$ T cells (Giannakis et al., 2002; Okada et al., 1995; Sweetser et al., 1998). Further, the type V pili of *Neisseria gonorrhoeae* and *Neisseria meningitidis* recognize an epitope on SCR3 and the region STP of CD46 (Kallstrom et al., 2001). A role for CD46 in the infection of urinal tract by uropathogenic *Escherichia coli* (*E. coli*) has also been suggested. C3b opsonised uropathogenic *E. coli* can exploit the binding of C3b to CD46 and infect human kidney tubular epithelial cells (Otero et al., 2006)**.** The highlighted studies underline CD46's role as a pathogen receptor and show that its engagement induces intracellular signals upon engagement. Particularly the observation that CD46 activation strongly modulates cytokine production by macrophages (Karp et al., 1996) paved the way for the notion that pathogen's may bind to CD46 because of its immunomodulatory function. The seminal discovery by Astier in 2000 that CD46 functions as a co-stimulatory molecule during human $CD4^+$ T cell activation (described in 1.4.2.3) then further increased the interest in the signalling capabilities of CD46 on immune competent cells (Astier et al., 2000).

1.5.2.3 CD46: a T cell co-stimulatory molecule

The most comprehensive data on CD46 signaling pathways have been described in T cells. Following CD46 crosslinking with antibodies on Jurkat cells, CD46-CYT-2 undergoes tyrosine phosphorylation by the Src kinase Lck (Taub et al., 1993b). In primary T $CD4^+$ cells, engagement of CD46 alone induces activation of the adaptor proteins p120CBL and LAT (Astier et al., 2000). Additionally, concurrent activation of TCR and CD46 leads to tyrosine phosphorylation of the guanine-nucleotide-exchange factor Vav, the GTPase Rac and the extracellular signal-regulated kinases ERK1/ERK2, important regulators of the MAPK pathway (Zaffran et al., 2001). The

functional consequences of the described signaling events are an increased proliferation of T cells (Astier et al., 2000) and a strong regulation of cytokine production (Kemper et al., 2003). High IL-10 secretion is indeed a feature of $CD4^+$ T-cell stimulated through TCR, CD46 and CD25 and the ability to suppress bystander $CD4^+$, $CD8^+$ (Kemper et al., 2003) and γδ T-cells (Truscott et al., 2010) highlights an important link between complement and adaptive immunity. Further studies have shown how TCR/CD46 engagement on T cell modulates the function of other immune cells including DCs and B cells. CD46-activated T cells secrete soluble CD40 ligand and granulocyte–macrophage colony-stimulating factor (GM-CSF) supporting DCs maturation (Barchet et al., 2006). Further, co-culturing B cells with CD46-induced T cells favors B cell production of immunoglobulins (Ig) IgM and IgG1 (Fuchs et al., 2009). The mechanisms underlying such boost in antibody production is not understood but it requires cell-cell contact and secretion of IL-10. Attesting for the importance of this mechanism are the findings that T cells from a CD46-deficient patient are impaired in promoting Ig production (Fuchs et al., 2009) and that circa 30% of CD46 deficient patients develop Common Variable Immunodeficiency (CVID), a syndrome characterized by hypo-gammaglobulinemia (Fremeaux-Bacchi et al., 2006). Recent data on the effects of CD46 activated T cells on antigen specific T cells revealed a mechanism of contact-independent suppression towards mycobacterial specific effector T cells. Interestingly the main soluble factor responsible for down modulation of effector $CD4^+$, $CD8^+$ and γδT cells was not IL-10 (Truscott et al., 2010). This study was the first to prove that supernatants from CD46 activated T cells do not have adverse effects on antigen-specific DCs confirming the previous finding of a positive influence of CD46 on DC maturation and antigen presentation (Barchet et al., 2006).

1.5.3 CD46 in Multiple Sclerosis

Multiple sclerosis (MS) is a neurological disease of that affects the central nervous system (CNS). The aetiology of MS is unknown but is thought to result from genetic and environmental risk factors (Ramagopalan et al., 2010). Damage to the CNS is mediated by auto-reactive T cells and this research area has been highly investigated. T-reg cells have been found to be impaired in MS patients, suggesting that T effectors

cells would be able to drive an uncontrolled immune response towards CNS derived auto-antigens (Haas et al., 2005). In line with these findings is the work by Astier et al., (2006) on CD46-induced T cells (Astier et al., 2006): these studies showed that CD46 mediated Tr-1 cells induced from lymphocytes of MS patients have a defect in the production of IL-10 when compared with healthy donors. Further analyses on the function of CD46 in MS patients showed how $CD4^+$ T cells are not able to suppress bystander effector T cells (Martinez-Forero et al., 2008). Confirming the previous studies are *in vivo* data obtained using a Cynomolgus monkey model of MS. T cells proliferation and IL-10 secretion, but not IFN-γ, were impaired upon CD46 activation of T cells derived from animals with Experimental Autoimmune Encephalomyelitis (Itoh et al., 1991). Patients with MS have been also investigated for DC function upon CD46 engagement (Vaknin-Dembinsky et al., 2008). IL-23 increased expression by myeloid DCs activated through CD46 was shown to be able to induce IL-17 secretion by T cells. IL-17 has been recently identified as a component responsible for the damage to CNS and T_H17 cells seem to have a pivotal role in driving MS. A recent study describing higher IL-17 production in MS derived T cells upon TCR and CD46 engagement (Yao et al., 2010) further suggests that CD46 signaling may be involved in the expansion of T_H17 cells in MS.

1.6 Hypothesis and aims of the thesis

The description of the signaling pathways triggered by the engagement of CD46 on $CD4^+$ T cells (Astier et al., 2000; Zaffran et al., 2001) shed light on the costimulatory properties of CD46 and uncovered an existing link between complement and T cell biology. The functional significance of CD46 signals in $CD4^+$ T cells was shown to be of an immunoregulatory nature with important links to human pathology (Astier et al., 2006; Kemper et al., 2001). In fact, high IL-10 secretion is indeed a feature of $CD4^+$ T-cells stimulated through TCR, CD46 and the IL-2 receptor and impairments in CD46-induced IL-10 production have been described in multiple sclerosis. Although the nature of CD46-activated T cells was found to be mainly immunosuppressive (Shalapour et al., ; Truscott et al., 2010) an important additional feature of these cells is the secretion of IFN-γ. The biological significance of this observation is not clear. In addition, from a translational perspective, the secretion of IFN-γ poses a main barrier for the potential usage of CD46-induced IL-10 secreting cells as a therapeutic tool to ameliorate graft rejection or autoimmunity and needs to be addressed.

Given the importance of both the specificity and the strength of signaling in the induction of T cell activation and cytokine secretion it was hypothesized that:

- CD46-induced pro-inflammatory (IFN-γ) and anti-inflammatory (IL-10) cytokines are differentially modulated by the strength of the inducing signals TCR, CD46, and IL-2 receptor.

Hence, the main objectives of this study were to: investigate the influence of the strength of activating signals on the secretion of IFN-γ and IL-10, understand whether specific signals could preferentially skew T cells towards IFN-γ or IL-10 production and identify pathways involved in such modulation.

The identification of a cellular switch mechanism between IFN-γ and IL-10 secreting cells, triggered by CD46 and IL-2 signals, prompted further analyses of the different cytokine secreting populations in CD46-induced T cells as it was postulated that crucial intermediaries in the regulation of such switch mechanism were likely to exist. The transcriptional signature of CD46-induced IFN-γ secreting cells was compared with the one of IL-10 secreting cells identifying a transcriptional profile consistently

observed across biological replicates. The asparagine endopeptidase (AEP) which was one of the most differentially expressed genes between IFN-γ and IL-10 producing cells, was investigated in order to gather whether this enzyme is a key mediator in the switch induced by CD46 or whether it impacts on the production/secretion of IFN-γ and IL-10.

The pathways triggered in CD46-induced IFN-γ and IL-10 producing cells are likely to impact *in vivo* not only bystander immune cells but also the surrounding tissues. In addition to the transcriptional signature of CD46-induced IFN-γ and IL-10 producing cells, which showed an up-regulation of inflammatory and tissue repair genes respectively, previous data generated from the laboratory of Dr. Kemper identified molecules involved in epithelial barrier integrity. The CD46 binding kinase, Ste20/SPS1-related serine/threonine kinase (SPAK), is important in T cell signalling. SPAK has also important functions in epithelial cells and plays key a role in epithelial cell barrier integrity (Yan, et al., 2007). Hence, it was postulated that CD46 signals may impact intestinal epithelium indirectly via T cell cytokines and growth factors and directly via CD46 signalling. In this study the consequences of CD46 activation on epithelial cells were assessed with the aim of describing the main consequences of CD46 signalling in intestinal epithelial cells to allow future studies on T cell-epithelial crosstalk.

Chapter 2

Materials and Methods

2.1 Cell sources

2.1.1 Blood and synovial fluid samples

T cells were obtained from blood samples of healthy volunteers under the approval of the South London Research Ethics Committee; reference number 09/H0804/72. Blood or synovial fluid from patients with rheumatoid arthritis was collected and processed with the approval of the Bromley Research Ethics Committee; reference number 06/Q0705/20. Healthy donors or patients with inflammatory arthritis (including rheumatoid arthritis and juvenile idiopathic arthritis) were recruited and informed consent was obtained from all subjects included in the study. Patients with rheumatoid arthritis were all female and of the age of 62 (RA patient 1), 39 (RA patient 2) and 54 (RA patient 3). The juvenile arthritis patient was female and 20 years old (Figure 3.14). All patients had a disease activity score for 28 joints of over 5.1, representing moderately severe activity, despite therapy with a combination of the disease-modifying anti-rheumatic drugs methotrexate, hydroxychloroquine and sulfasalazine. Synovial fluid was obtained during therapeutic arthrocentesis of knee joints performed by the leading clinician and collaborator, Professor Andrew Cope (KCL). Healthy donors (HD) were all female and of the age of 31 (HD1), 37 (HD2) and 33 (HD3) (Figure 3.14).

2.1.2 Caco-2 cell line

The Caco-2 (HTB-37) cell line, derived from human colorectal adenocarcinoma was obtained from the American Tissue Culture Collection (ATCC) and cultured as per manufacturer’s protocol.

2.1.3 Bacteria

The uropathogenic *E.coli* strain J96 is a serum-resistant, hemolysin-secreting strain that expresses type 1 and P fimbriae and was obtained from ATCC (clone 700336). J96 bacteria were grown in Luria-Bertani broth (Sigma Aldrich, Saint Louis, USA) and bacteria numbers assessed by photospectometry at 600 nm.

2.2 Tissue culture media

2.2.1 T cell medium

$CD4^+$ T cells and Jurkat T cells were maintained in RPMI 1640 medium (HyClone, Thermoscientific, Massachusetts, US) with 10% foetal calf serum (HyClone, Thermoscientific, Massachusetts, US), 100U/ml penicillin, 100 mg/ml streptomycin and 2 mM GlutaMAX (all from GIBCO, US). Serum was heat inactivated at 56°C for 30 min, aliquoted and stored at -40°C. Before use all components of tissue culture media were filter sterilised and stored at 4°C.

2.2.2 Caco-2 cell medium

Caco-2 cells were cultured in Eagle's Minimum Essential Medium with 20% FCS, 100U/ml penicillin, 100 mg/ml streptomycin and 2mM GlutaMAX. Serum was heat inactivated at 56°C for 30 min and stored at -40°C. Before use all the components of tissue culture media were filter sterilised and stored at 4°C.

2.3 Antibodies

2.3.1 Activating and neutralizing antibodies

Cell-stimulating antibodies were purified from a specific hybridoma line (anti-CD3; OKT-3) or purchased from BD Biosciences (anti-CD28; CD28.2). The monoclonal antibody to CD46, TRA-2-10, recognizes an epitope in the first repeat of the complement control protein repeats (Liszewski et al., 2000) and was generated in house (Wang et al., 2000). The monoclonal antibody to human CD46, GB24 binds to complement control protein repeats 3 and 4 (Liszewski et al., 2000), was generated in house and kindly provided by John Atkinson. The function-neutralizing monoclonal antibodies to human IL-2 (MQ1-17H12), IL-4 (8D4-8), IL-10 (JES3-9D7), IL-12 (C8.6) and IFN-γ (4S.B3) were purchased from BD Pharmingen.

2.3.2 Antibodies used for flow cytometry staining, confocal microscopy and Western blot

Surface, intracellular staining and Western blot analyses for specific marker/protein expression were performed using the antibodies in Table 2.1. Antibodies that required fluorochrome labelling were directly conjugated using the Zenon Mouse R-phycoerythrin Mouse IgG_1 Labeling kit (Invitrogen, Paisley, U.K.). The monoclonal antibody to human ICER/CREM was labelled with phycoerythrin using the Zenon labeling kit. According to the protocol, 1 μg of antibody was incubated with 5 μl of the Zenon mouse IgG labelling reagent for 5 min. Following the incubation time, 5 μl of the Zenon blocking reagent were added and the mixture was incubated for 5 min. The complex was used for cell staining within 30 min of labelling.

Table 2.1 Antibodies used for flow cytometry staining, confocal microscopy and immunoblotting

Target	Isotype	Clone	Fluorophore	Supplier
AEP	Rabbit polyclonal IgG	ab47146	Unconjugated	Abcam
β-actin	Mouse IgG_1	mAbcam8226	Unconjugated	Abcam
CD3	Mouse IgG_1	HIT3a	PE	BD
CD4	Mouse IgG_1	RPA-T4	PE	BD
CD4	Mouse IgG_{2a}	RPA-T4	APC	BD
CD4	Mouse IgG_1	RPA-T4	Pacific Blue	BD
CD25	Mouse IgG_1	M-A251	FITC	BD
CD25	Mouse IgG_1	M-A251	APC	BD
CD45RA	Mouse IgG_{2b}	HI100	FITC	BD
CD45RA	Mouse IgG_{2b}	HI100	PE	BD
CD45RO	Mouse IgG_{2a}	UCHL1	FITC	BD
CD45RO	Mouse IgG_{2a}	UCHL1	APC	BD
CD46	Mouse IgG_{2k}	E4.3	FITC	BD
CD69	Mouse IgG_1	FN50	APC	BD
CD197 (CCR7)	Mouse IgG_{2a}	150503	Cy7	BD
Foxp3	Mouse IgG_1	236A/E7	PE	BD
Granzyme B	Mouse IgG_1	GB11	FITC	BD
ICER/CREM	Mouse IgG_1	ab54625	Unconjugated	Abcam
IFN-γ	Mouse IgG_1	4S.B3	FITC	BD
IL-10	Rat IgG2a	JES3-19F1	APC	BD
LAMP1	Mouse IgG_1	ab25630	Unconjugated	Abcam
SPAK	Rabbit polyclonal	#2281	Unconjugated	Cell Sign. Tec.*
Stat4 (pY693)	Mouse IgG_1	38/p-Stat4	PE	BD
Stat5 (pY694)	Mouse IgG1	47	PE	BD
Stat6 (pY641)	Mouse IgG2a	18/P-Stat6	PE	BD

* Cell Signaling Technology

Table 2.2 Secondary Antibodies

Target	Isotype	Dilution	Format	Supplier
IgG	Rabbit polyclonal	1:2000	HRP conjugated	GE Healthcare
IgG	Mouse polyclonal	1:2000	HRP conjugated	GE Healthcare
IgG	Mouse polyclonal	1:200	Alexafluor 488 conjugated	Invitorgen
IgG	Rabbit polyclonal	1:200	TRITC conjugated	Southern Biotech

2.4 Recombinant proteins

2.4.1 Recombinant cytokines

Recombinant human IL-4, IL-10, IL-12 and IFN-γ (for in vitro T_H1 or T_H2 skewing) were from BD Biosciences. Recombinant human IL-2 was kindly provided by Dr. Christine Pham (Washington University, Saint Louis, USA).

2.4.2 Soluble CD46

Soluble CD46 was generated by cloning of cDNA encoding short consensus repeats 1–4 of human CD46 into the pET15-b vector (Merck, Nottingham) by Dr. Teresa Melchionna and Dr. Richard Smith (King's College London). BL21 DE3 bacteria (Merck) were transfected with the construct and recombinant soluble CD46 was then purified from the inclusion bodies and refolded according to a published method (White et al., 2004).

2.5 Purification of $CD4^+$ T cells and $CD4^+$ T cell subpopulations

2.5.1 Purification of PBMCs from whole blood

PBMCs were isolated from buffy coats, leukocyte cones or blood samples from healthy volunteers by using gradient centrifugation with Ficoll Histoplaque (GE Healthcare; Waukesha, Wisconsin). Blood was diluted 1:1 with PBS 1X (HyClone, Thermoscientific, Massachusetts, US) and layered on 15 mL of Ficoll. The preparation was then centrifuged at 1600 RPM for 30 min without brakes. Following the centrifugation step the leukocyte layer was harvested by aspiration, washed 3X in PBS, then subjected to cell isolation using magnetic cell sorting.

2.5.2 Isolation of whole $CD4^+$ T cells from PBMCs via magnetic cell sorting (positive selection)

$CD4^+$ T lymphocytes were isolated from PBMCs or synovial fluid with CD4 MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. In brief, PBMCs were washed in PBS and centrifuged for 10 min at 1400 RPM counted and resuspended in 80 µl of bead buffer per 10^7 cells. CD4 microbeads were added using 20 µl per 10^7 cells and the mixture was incubated at 4°C for 15 min. Cells were then washed using 25 ml of bead buffer, filtered through a 70µM cell strainer (BD, Bedford, USA) and applied onto an LS column (Miltenyi Biotec) attached to a magnet. T cells retained in the column were washed 3 times with 3 ml of bead buffer and subsequently eluted by removing the LS column from the magnet and applying 3 ml of bead buffer to the column. The purity of isolated $CD4^+$ T cells was routinely evaluated by FACS analysis as approximately 99% using the gating strategy shown in Figure 2.1. Total $CD4^+$ cells were then counted and activated accordingly.

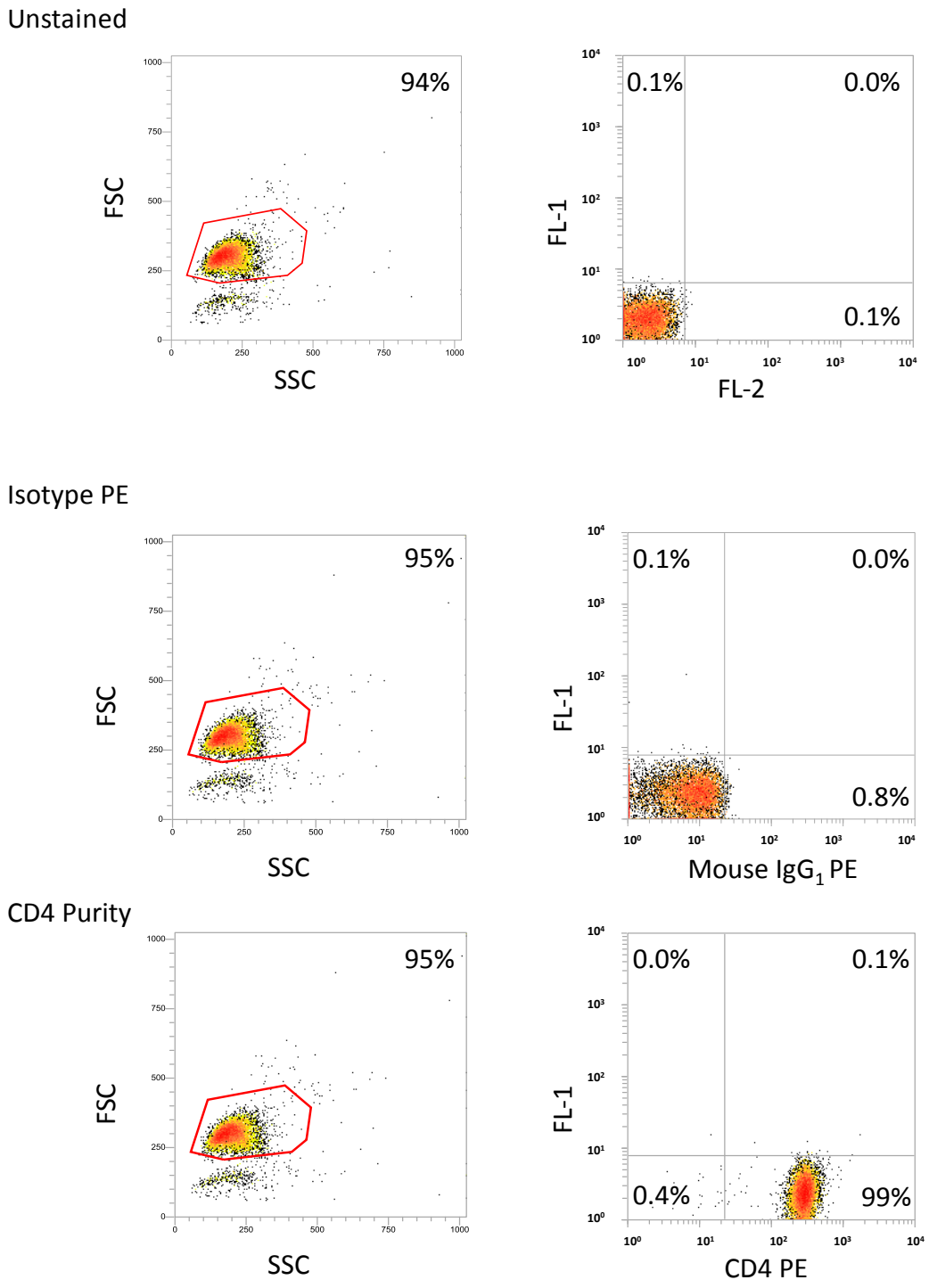


Figure 2.1 Gating strategy and staining controls of purified CD4$^+$ T cells. Following magnetic sorting via positive selection, T cells were gated according to the FSC and SSC profile. Purity of CD4$^+$ T cells was verified by surface staining with an anti-CD4 antibody. Representative gating strategy with unstained T cells and isotype control antibody staining. Values shown represent the percentage of positive cells within the relevant FSC/SSC gate or FL-1 vs FL-2 quadrant.

2.5.3 Isolation of naïve $CD4^+$ T cells ($CD45RA^+/CD45RO^-$) via magnetic labelling (negative selection)

Isolation of naïve T cells via negative selection from PBMCs was carried out following the Miltenyi Biotec instructions. PBMCs were washed in PBS and centrifuged for 10 min at 1800 RPM counted and resuspended in 40 µl of bead buffer per 10^7 cells. The naïve $CD4^+$ T Cell Biotin-Antibody cocktail II was added (10 µl per 10^7 cells). Cells were mixed and incubated for 15 min at 4°C. Cells were then washed using 1 ml of bead buffer per 10^7 cells. Cells were centrifuged and then resuspended in 80 µl of bead buffer per 10^7 cells. Anti-Biotin microbeads were added (20 µl per 10^7 cells) and cells were incubated for 15 min at 4°C in the dark. Following incubation, cells were resuspended in 500 µl of buffer per 10^7 cells and filtered through a 70 µM cell strainer (BD, Bedford, USA). Cells were then applied onto an LS column (Miltenyi Biotec) and the flow-through containing the naïve subset of $CD4^+$ T cells was collected. The purity of isolated naïve T cells was routinely assessed by FACS analysis as approximately 98%. FACS analysis was performed using the antibodies and the gating strategy shown in Figure 2.2.

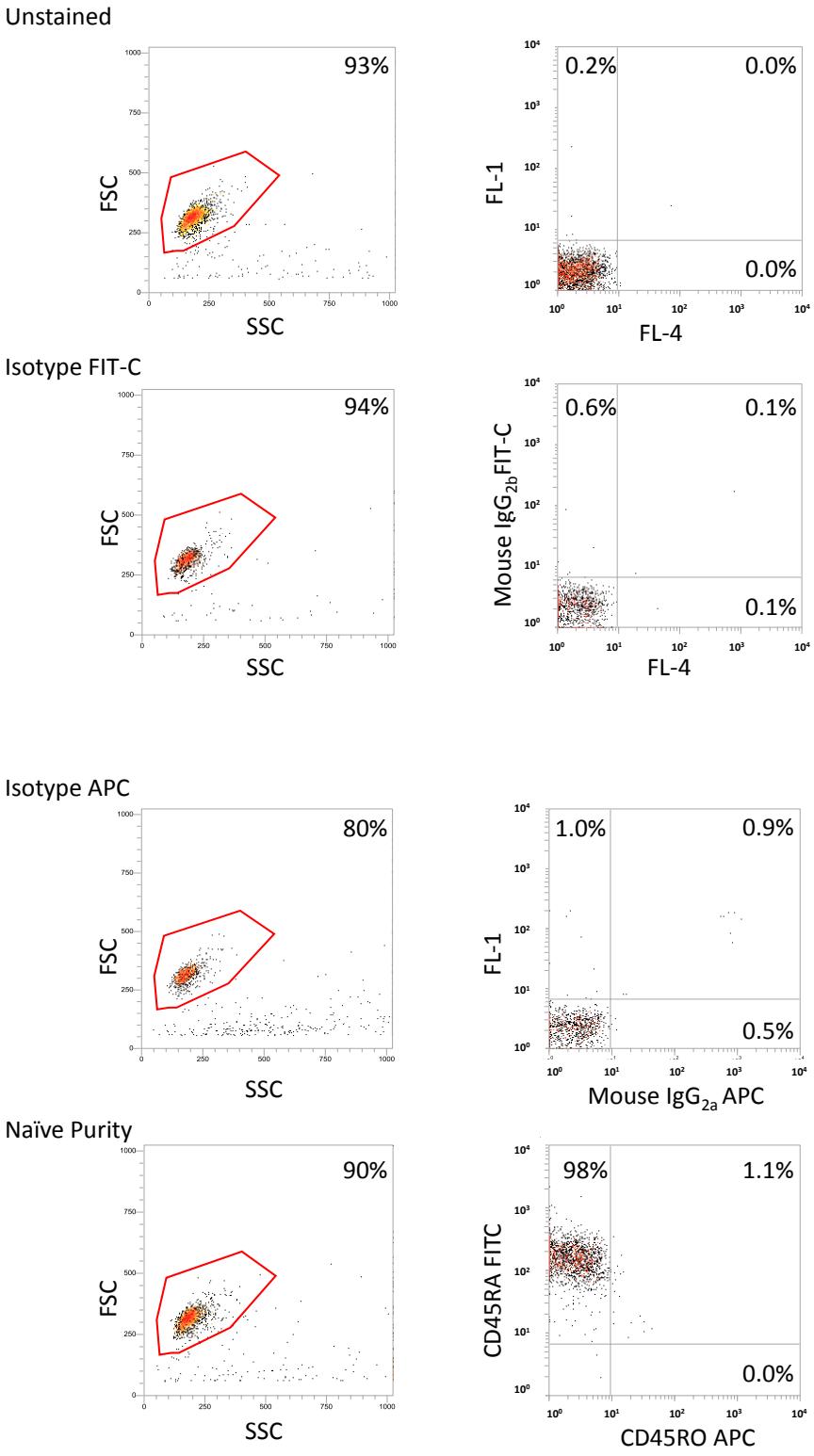


Figure 2.2 Gating strategy and staining controls of purified naïve (CD45RA+/CD45RO-) lymphocytes. Following magnetic sorting via negative selection, the purity of naïve T cells was assessed by surface staining with antibodies to CD45RA (FITC) and CD45RO (APC) and matched isotype control antibodies. T cells were gated according to the FSC and SSC profile. Values shown represent the percentage of positive cells within the relevant FSC/SSC gate or staining quadrant.

2.5.4 Isolation of naïve $CD4^+$ T cells ($CD45RA^+/CD45RO^-$) via cell sorting

To purify naïve lymphocytes through FACS sorting whole $CD4^+$ T cells were initially sorted from peripheral blood via magnetic cell sorting with CD4 Miltenyi beads (positive selection). Subsequently, $CD4^+$ lymphocytes were resuspended in FACS buffer at a concentration of 30 x 10^6 cells per 500µl and surface stained with antibodies to CD4, CD45RA, CD45RO, CD25 and CCR7 (Figure 2.3). Staining was carried out at 4°C for 30 min in the dark. Lymphocytes were then washed twice in FACS buffer, resuspended in media at a concentration of 30 x 10^6 cells per ml. Sorting was performed on a BD FACSAria™ II Cell Sorter using the gating strategy in figure 2.3. The purity of isolated T cells was verified post-sort and was approximately 97% for $CD45RA^+$ T cells (Figure 2.3b).

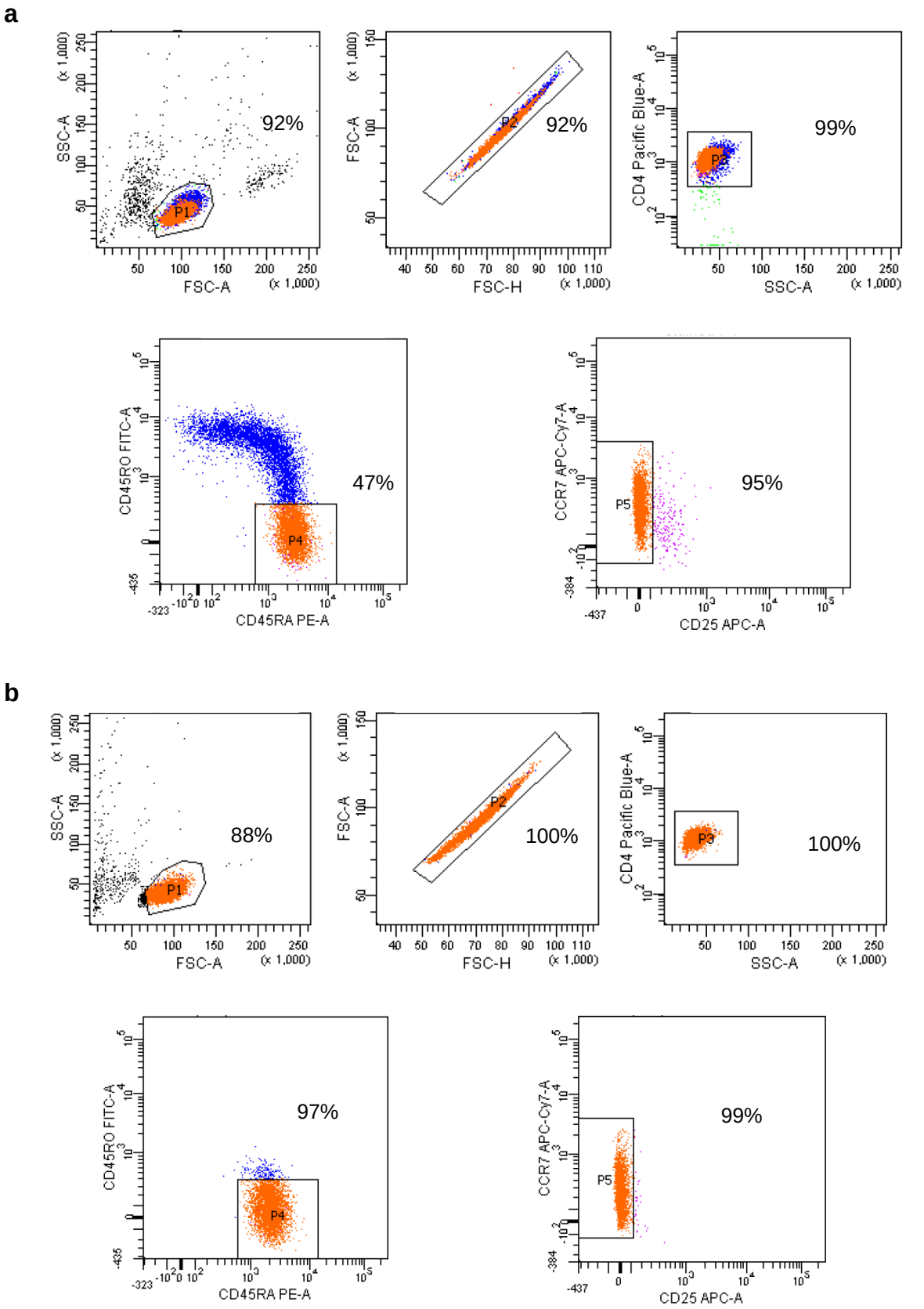


Figure 2.3 Sorting strategy of CD4$^+$, CD45RA$^+$, CD45RO$^-$, CD25$^-$ and CCR7$^+$ naïve T cells. Following a CD4 positive selection of T cells with Miltenyi microbeads from peripheral blood mononuclear cells, T cells were surface stained for sorting using CD4, CD45RA, CD45RO, CD25 and CCR7 antibodies. a) Pre-sort gating strategy of CD4$^+$ cells based on FSC/SSC profile (P1, upper left dot plot), doublet depletion (P2,

upper central dot plot), CD4+ cells (P3, upper right dot plot), CD45RA+/CD45RO- cells (P4, lower left dot plot), CD25- and CCR7+ cells gates (P5, lower right dot plot). b) Post-sort purity was verified by analysing 10.000 events. Values shown refer to the percentage of positive cells within the relevant gate.

2.6 T cell activation and gating strategy

Purified $CD4^+$ T cells were activated in 96 or 48-well culture plates (Iwaki, Japan). Wells were coated overnight at 4°C with mAbs to CD3, CD28 and/or CD46 at a concentration of 2.0μg per ml of PBS. Each well was coated with 75 μl in the case of 96-well plates and 125 μl in the case of 48-well plates. Post purification T cells were resuspended in media, containing recombinant human IL-2 (rhIL-2), with concentrations varying between 1 and 100 Units/ml, according to the experiment. Cells were then seeded at a concentration of 1.5×10^5 (96-well plates) to 3.5×10^5 (48-well plates) cells per well. Following activation cells were stained according to the experiment requirements. Non-activated or activated $CD4^+$ T cells were gated according to the FSC and SSC profile in order to include blasting cells and exclude dead cells from the analysis. Propidium iodide staining was used to assess cell viability within the FSC and SSC gate (Figure 2.4). The percentage of non-viable cells within the FSC/SSC gate was < 4% for non-activated and activated cells.

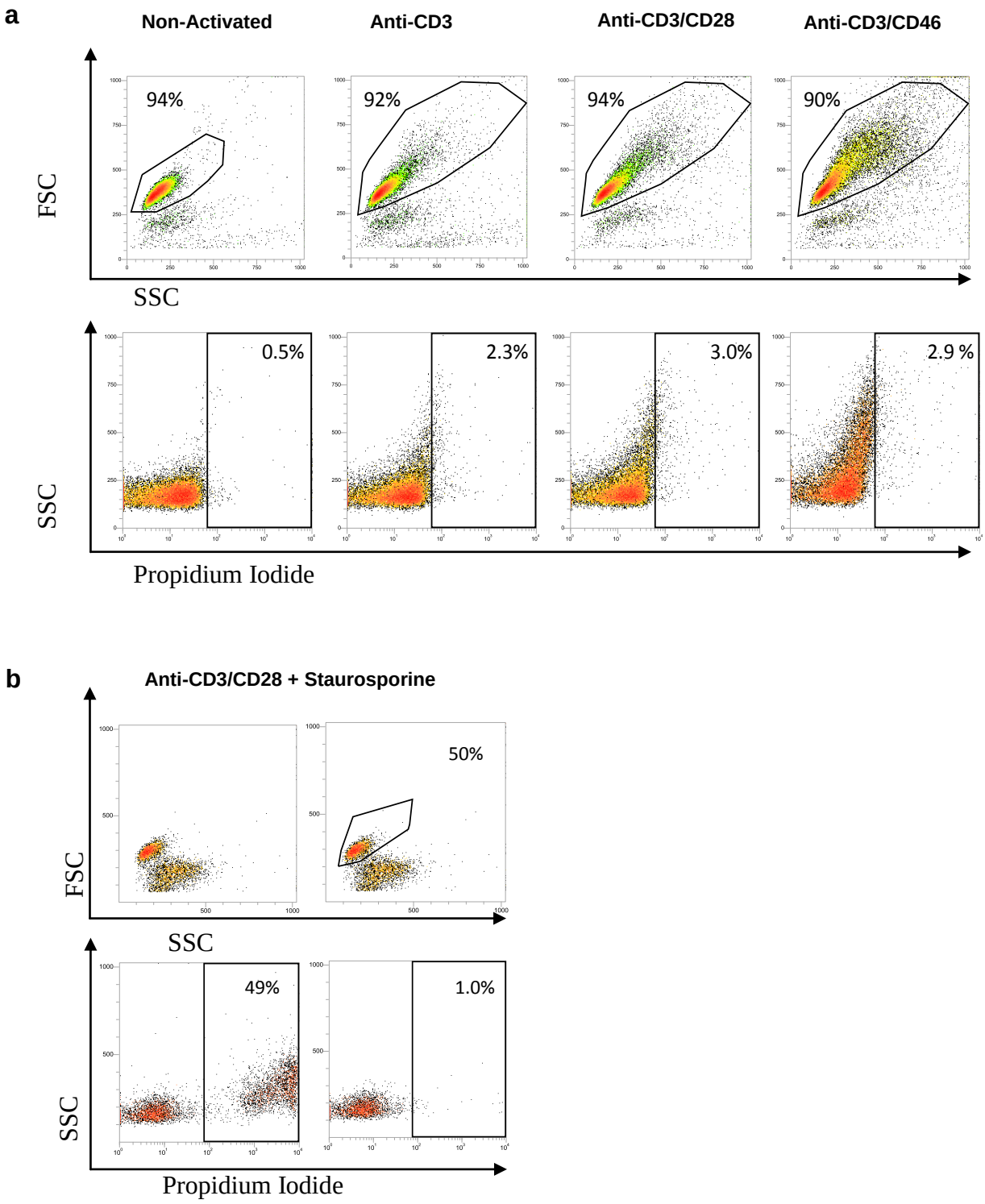


Figure 2.4 Gating strategy and viability of non-activated and activated CD4$^+$ T cells. Purified CD4$^+$ T cells were plated in a 48 well plate coated with activating antibodies to CD3, CD28 or CD46 at a concentration of 2 µg/ml. Cells were activated for 36 hours in the presence of 50 U/ml of rhIL-2. Following activation CD4$^+$ T cells were harvested, washed twice with FACS buffer and stained with propridium iodide (PI) to discriminate non-viable cells within the FSC/SSC gate. b) Anti-CD3/CD28 activated T cells were activated as in (a) and treated with 100µM of Staurosporin. Viability was assessed in non-gated (left dot plots) and gated (right dot plots) cells

through PI staining. Values are indicative of the percentage of positive cells within the relevant gate.

2.7 T_H1 and T_H2 skewing of naïve $CD4^+$ T cells

T_H1 and T_H2 cells were generated from sorted naïve $CD4^+$ T cells as follows: naïve T cells were activated through plate-bound mAb to CD3 and mAb to CD28 (2 μg/ml each) at a concentration of 0.5-1x10^6 cells per well in 48-well plates. T_H1 skewing media included function-neutralizing mAb to IL-4 (10 μg/ml) and recombinant human IL-12 (10 ng/ml). T_H2 skewing media contained anti-IFN-γ (10 μg/ml) and recombinant human IL-4 (20 ng/ml). Following 48-72 hours of activation cells were expanded by transfer in 24 well plates coated with mAb to CD3 and mAb to CD28 (2 μg/ml each). Skewing media was added according to T_H1 and T_H2 induction in addition to rhIL-2 (5 U/ml). Expansion was carried out for a maximal period of 13 days before cytokine staining and marker analysis of successful T_H1 (IFN-γ and T-bet positive) and T_H2 (IL-4 and GATA3 positive) generation.

2.8 Caco-2 culture and activation

Cells were cultured in 48-well plates (3 x 10^4 cells/well) (Iwaki, Asahi Glass Co., LTD., Japan), 24 trans-well systems (polycarbonate membrane, pore size 0.4 μm, membrane insert Ø 12 mm; Corning, USA) or in wells provided by the CytoSelect™ 24-Well Wound Healing Assay (Cell Biolabs Inc., USA) for 6-7 days minimum to a fully confluent monolayer. Cells were initially seeded at a concentration of 3 × 10^4 (48-well plates) or 5 × 10^4 (24-well plates) cells per well. Epithelial cell layer integrity (i.e. formation of functional tight and adherens junctions) was monitored by trans epithelial resistance measurement using the epithelial voltohmmeter EVOM2 (World Precision Instruments Inc.,Sarasota, FL, USA). When TER values reached a minimum of 1000 Ω/cm2, mAbs to CD46, an isotype control mAb (10 μg/ml), or EtOH (final concentration of 15%) was added and TER measured at different time points post activation.

2.9 Assays measuring T cell viability and proliferation

2.9.1 Viability assay

$CD4^+$ T cell viability was tested using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), a colourimetric method for determining the number of viable cells in proliferation. The conversion of MTS into soluble formazan is accomplished by viable cells and is measured by the absorbance at 490 nm. $CD4^+$ T cells were plated in 96 well plates at a concentration of 1-1.5 x 10^5 per well. CellTiter 96 Aqueous One Solution Reagent was added using 20 µl per well and cells were incubated between 1 and 4 hours at 37°C (5% CO_2). The absorbance was then measured at λ=490nm on a SPECTRA max plus reader (Molecular Devices, Berkshire, UK).

2.9.2 Suppression assay

The suppressive abilities of supernatants collected from activated T cells were evaluated using the viability assay (paragraph 2.9.1). $CD4^+$ T cells were plated in 96-well plates at a concentration of 1.0 x 10^5 per well. Cells were activated through plate-bound mAb to CD3 and mAb to CD28 (2 µg/ml). Supernatants were added using a volume of 100 µl per well and cellular proliferation was tested at day 6 through the viability assay.

2.9.3 Proliferation assay

Cellular proliferation of T cells activated in the presence of carrier solution or AEP inhibitor was assessed by identifying cellular division cycles through 5-(and 6) Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. Purified $CD4^+$ T cells were pelleted at 1400 RPM for 10 min and washed twice in PBS. The CFSE solution was prepared by diluting CFSE (Molecular probes, Invitrogen) in PBS to a final concentration of 2.5 µmol/L. Lymphocytes were resuspended in CFSE solution at a concentration of 10^7 cells per ml of CFSE solution and incubated in the dark at room temperature for 3 min. Lymphocytes were then washed in complete medium,

centrifuged at 1400 RPM for 10 min and resuspended in the appropriate medium before experimental activation.

2.10 Methods for protein expression, quantification and interaction

2.10.1 T cell staining and flow cytometry

Surface and intracellular staining of activated or non-activated T cells was performed in order to analyse the expression and/or phosphorylation status of proteins of interest on the cell surface or within the cytoplasm. Surface staining was either carried out alone or in conjunction with intracellular staining. The BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, San Diego, USA) was used for surface and intracellular staining accordingly; purified $CD4^+$ T cells were washed twice in PBS and resuspended in staining buffer (PBS, 1% FCS) at a concentration of 10^6 per 50 µl of staining buffer. Surface staining was carried out at 4°C for 30 min. Cells were then washed twice with staining buffer and fixed by resuspending them into the 1X BD Perm/Wash buffer solution for 20 min at 4°C. Cells were then washed twice in 1X BD Perm/Wash buffer and resuspended in 50 µl of BD Perm/Wash buffer containing an optimal concentration of antibodies for staining. Staining was carried out at 4°C for 30 min in the dark. Cells were then washed with 1X BD Perm/Wash buffer and resuspended in staining buffer for flow cytometry analysis. Flow cytometry was carried out on a BD FACSCalibur™ (BD Biosciences, San Jose, USA) and data was analysed using the Venturione software (Sheffield, UK).

2.10.2 Measurement of Foxp3 expression

The Foxp3 staining was carried out using the FOXP3 Fix/Perm Buffer Set from Biolegend (San Diego, USA) according to the manufacturer's protocol. To each sample containing 10^5-10^6 cells, 1 ml of the BioLegend FOXP3 Fix/Perm solution was added. Samples were mixed and incubated in the dark for 20 min at room temperature. Cells were then washed once in PBS and subsequently in the 1X BioLegend's FOXP3 Perm buffer. Samples were then incubated at room temperature in the dark for 15 minutes and spun down. The pellets were resuspended in 100 µl of 1X BioLegend's

FOXP3 Perm buffer. Anti-human Foxp3 (table 2.1) was added, using 3 µl per sample and cells were incubated for 1 hour in the dark. After washing the samples twice in PBS staining was analysed on the FACScalibur (BD Biosciences).

2.10.3 Assessment of the phosphorylation status of signal transducers and activators of transcription (STAT) proteins

Staining for phospho-STAT4, phospho-STAT5 and phospho-STAT6 was carried out using BD anti-pSTAT4, anti-pSTAT5 and anti-pSTAT6 (Table 2.1). The intracellular phospho-staining was performed using BD Phosflow Perm Buffer III and diluted Permfix Phosflow buffer. The phospho-staining was performed according to the manufacturers' protocol (Alternative protocol 2; BD); following surface staining, cells were fixed by adding 10 volumes of pre-warmed Lyse/Fix Buffer to the samples and incubated at 37°C for 10 min. Cells were then centrifuged at 1800 RPM for 6 min. Supernatants were removed, cells were resuspended and washed in PBS with an equivalent volume to the one of the Lyse/Fix Buffer. The pellet was washed as described above and cells were permeabilized by incubation on ice for 30 min in Perm Buffer III. Cells were then washed twice in Staining Buffer and resuspended in 250 µl for FACS analysis.

2.10.4 Confocal microscopy for asparagine endopeptidase (AEP) expression and localization

Intracellular staining of T cells was performed in order to analyse the expression and localization of the AEP protein through confocal microscopy. $CD4^+$ T were fixed and permeabilized with the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, San Diego, USA) as in 2.10.1. Following permeabilization primary antibodies were added for 1 hour at room temperature at a dilution of 1:100. Cells were then washed twice and resuspended in Perm 1X solution. Secondary antibodies were added at a dilution of 1:200 and samples were incubated for 1 hour. Cells were then washed twice in 1X BD Perm/Wash and centrifuged on slides using a cytocentrifuge.

The nuclear die, Dapi-Fluoromount-G (SouthernBiotech, USA) was added before samples were analysed on the A1R Si Confocal microscope (Nikon, Japan).

2.10.5 Measurement of protein expression by Western blot

2.10.5.1 Purification of proteins from sub-cellular compartments

Protein extraction from isolated nuclei and cytosolic fractions of either unstimulated or activated T cells was carried out using the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, Thermoscientific, Massachusetts, USA). Activated $CD4^+$ T cells were harvested and washed with PBS twice. The cell pellet was then lysed by adding Cell lysis buffer to pelleted cells (e.g. 100 µl of CER I for 10 µl cell pelleted volume). Cells were then vortexed for 10 sec, set on ice for 10 min and vortexed again. The cytoplasmic fraction was separated from nuclei by centrifuging at 3500 RPM for 7 min at 4°C. The supernatant containing the cytosolic protein extract was then transferred to a new tube and cleared by centrifuging at 16,000 RPM for 15 min at 4°C. The supernatant was transferred in a new labelled tube and stored at -80°C. The nuclei pellet was washed once using 500 µl of Nuclei Washing buffer, vortexed and set on ice for 2 min. Nuclei were then centrifuged for 7 min at 3500 RPM at 4°C. The supernatant was removed and 100-150 µl of the Nuclei storage buffer was used to resuspend the pellet containing the nuclei protein extract which was stored at -80°C.

Given that the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit was discontinued in June 2011, protein extraction was then carried out using the NE-PER kit (Thermo scientific, Massachusetts, USA). Harvested cells were washed twice in PBS and 1-10x 10^6 cells were then transferred in a 1.5 ml tube and pelleted at 3500 RPM for 3 min. Ice-cold CER I buffer was added to the cell pellet (e.g. 100 µl of CER I for 10 µl cell pelleted volume) vortexed for 15 sec and incubated on ice for 10 min. Ice-cold CER II buffer was added (5.5 µl of CER II for a starting pellet of 10 µl of volume) and cells were vortexed for 5 sec, then centrifuged for 5 min at 16.000 RPM. The supernatant was transferred to a chilled tube and stored at -80°C. The pellet was resuspended on ice-cold NER buffer and vortexed for 15 sec every 10 min for 5 times and then centrifuged at 16.000 RPM for 10 min. The supernatant was then transferred to a pre-chilled tube and stored at -80°C.

2.10.5.2 Protein quantification

Protein quantification was performed using the Bradford assay reagent (Sigma Aldrich, USA) and reporting the absorbance values of unknown samples to a standard curve generated with bovine albumin serum (BSA) (Sigma Aldrich, St. Louis, USA). The assay was performed in 96-well plates using 5 μl of a 0.1–1.4 mg/ml protein sample. Protein standards were prepared in buffer (PBS or ddH2O) ranging from 0.1–1.4 mg/ml using a BSA standard. Protein standards and samples were added in triplicate to separate wells using 5 μl of material per well. To the blank wells, 5 μl of buffer was added. To each well being used, 250 μl of the Bradford reagent were added and mixed on a shaker for 30 sec. Samples were then incubated 5 to 45 min and the absorbance at (λ= 595 nm) was recorded.

2.10.5.3 Western blot methodology

Protein samples were denatured by heating samples at 95°C for 5 min. Lane reducing or non-reducing marker (ThermoScientific, Rockford, USA) was added to samples at a dilution of 1:5. Samples were separated using precast polyacrylamide NuPAGE Bis-Tris Gels gels 4-12% (Life technologies, New York, USA) with TBE 1X buffer (see Buffers) at 150 Volts using a Bio-Rad basic PowerPac (Bio-Rad, California, USA) and transferred to the nitrocellulose membrane. The iBlot system (Invitrogen) was used for the transfer of proteins onto a nitrocellulose membrane which was then incubated in blocking buffer (5% milk in TBS-Tween) for 1 hour at 4°C under agitation. After blocking, the membrane was rinsed twice for 10 seconds in TBS-T (see Buffers). After the second wash the membrane was incubated with the primary antibody (dilution range 1:3.000 to 1:5000) in blocking buffer overnight. The membrane was then washed and the horseradish peroxidase (HRP)-conjugated secondary antibodies were added for 12 hours (Biosciences, NJ, USA). Detection was performed using the West Pico Luminol Enahncer Kit (ThermoScientific, Rockford, USA) and the exposure of the membrane to radio film with its subsequent development in a CompactX2 (Xograph Healthcare Ltd, Xograph House, Gloucester, UK).

2.10.6 Co-Immunoprecipitation

Caco-2 cells were cultured for 7 days in 24-well plates. A mAb to CD46 (TRA-2-10) or an isotype control mAb was added at 10 μg/ml for 3 hours. Cells were then lysed in lysis buffer (1% v/v maltoside, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 10% glycerol), and protease and phosphatase inhibitors (Sigma Aldrich). Lysates were incubated with a mAb GB-24 (anti-CD46) or an isotype-matched control mAb covalently coupled to Sepharose (Amersham) overnight at 4 °C. After washing, bound proteins were eluted, quantitated and analyzed by Western blotting under reducing conditions.

2.11 Cytokine measurement assays

2.11.1 Intracellular FACS staining

Intracellular protein expression was assessed by FACS staining either alone or in combination to surface staining according to the protocol in paragraph 2.11.1.

2.11.2 Secretion assay

Active single cell cytokine secretion was analysed using the Miltenyi Biotec Secretion Assay Kits according to the manufacturer's protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly for 10^6 cells, 1 mL of cold buffer was used for washing and centrifuging cells at 1600 RPM for 10 min at 4°C. The cell pellet was resuspend in 90 µl of cold medium and 10 µl of catch reagent per 10^6 cells (e.g. IFN-γ catch reagent) were added. Samples were kept 5 min on ice. For two colour cytokine analysis 20 µl of pre mixed catch reagent (e.g. IFN-γ and IL-10) per 10^6 cells were added to each sample and incubated on ice for 5 min. Warm media was then added I order to have 10^6 cells per mL of media and samples were incubated at 37°C for 45 min under slow continuous rotation. Subsequently cells were washed in cold buffer, resuspended in 90 µl of buffer per 10^6 cells and labelled by adding 10 µl per sample of the FITC, PE or APC conjugated detection antibody. Cells were incubated on ice for

10 min, washed by adding 2 ml of cold buffer and resuspended in 250 µl of cold buffer for flow cytometry analysis.

2.11.3 Cytokine Bead Array (CBA) assay

To quantify the amounts of IL-2, IL-4, IL-5, IL-10, TNF and IFN-γ secreted by activated T cells, the BD CBA Human Th1/Th2 Cytokine kit from Becton Dickinson was used according to the manufacturer's suggestions. Each capture bead was resuspended and a 10 µl aliquot per sample was added to the master mix. After vortexing, 50 µl of the master mix were added to 50 µl of each standard dilution and unknown samples. Further 50 µl of the human Th1/Th2 PE detection reagent was then added to the tubes and incubated for 3 hours. Standard dilutions and unknown samples were then washed with 1 ml of wash buffer and centrifuged at 1400 RPM for 5 min. Beads were then resuspended in 300 µl of washing buffer and acquired through FACS (Figure 2.5 and 2.6). Subsequently, standard curves were generated for analysis using BD Fcap array software or the BD Excel analysis software (Figure 2.6b and Appendix).

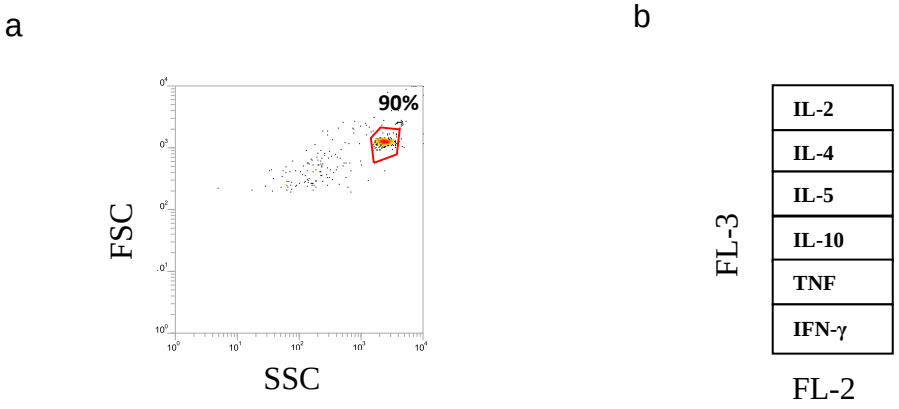
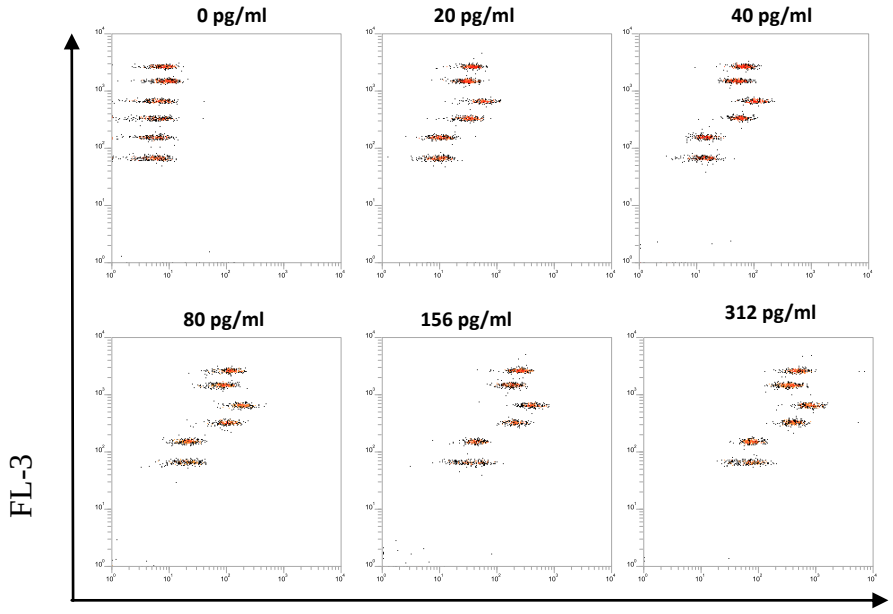
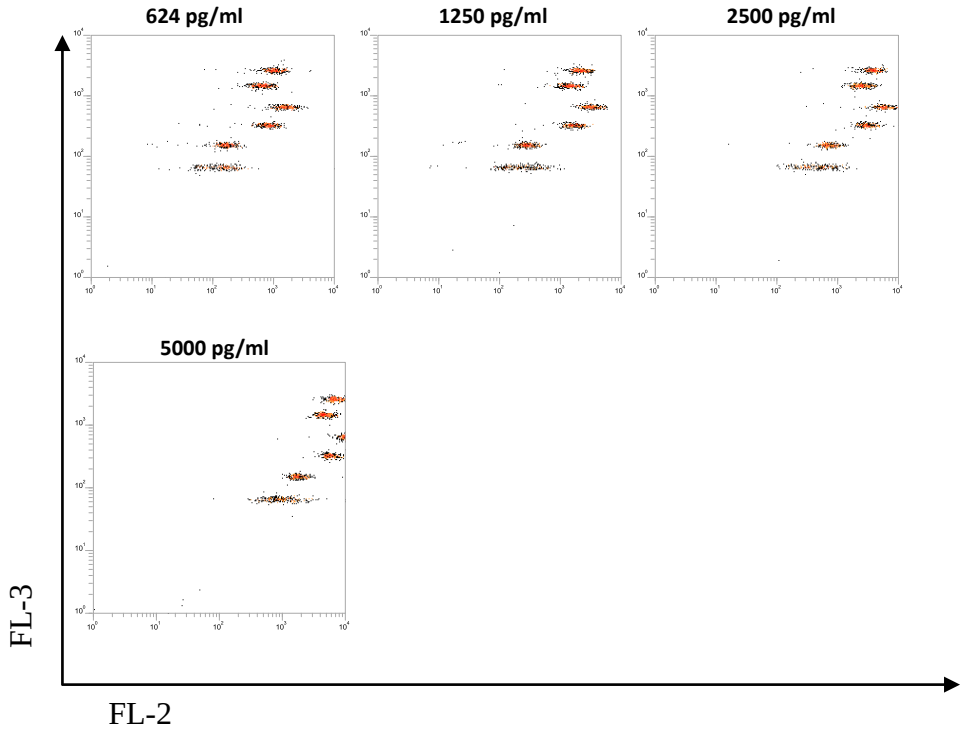


Figure 2.5 FSC/SSC gating strategy for the identification of the BD CBA beads. For each standard or unknown sample 1800 events were acquired within the FSC/SSC gate. Value is indicative of the percentage of beads within the gate. b) Diagram representing the cytokine associated with each bead population in an FL-3 vs FL-2 plot.

a


0 pg/ml
20 pg/ml
40 pg/ml
80 pg/ml
156 pg/ml
312 pg/ml
FL-3
FL-2


624 pg/ml
1250 pg/ml
2500 pg/ml
5000 pg/ml
FL-3
FL-2

b

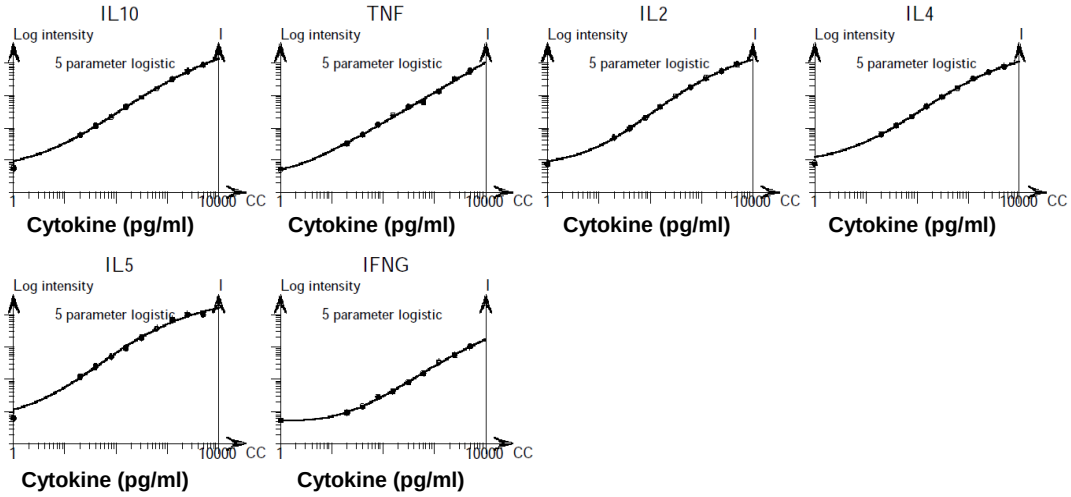


Figure 2.6 Standard curve generation for cytometric bead array analysis. a) Standard curves were obtained by serial 1:2 dilutions starting from the CBA top standard (5000pg/ml) in order to obtain the following concentrations: 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312 pg/ml, 156 pg/ml, 80 pg/ml, 40 pg/ml and 20 pg/ml. The 0 pg/ml standard was obtained by mixing 50 µl of the BD assay diluent with 50 µl of the master mix and 50 µl of the Th1/Th2 PE detection reagent. a) Dot plots relative to the fluorescent intensities for each standard bead. b) Standard curves generated for each bead through Fcap array software. Each standard curve had an R square value of fitting accuracy > than 99.86% (Appendix). IFNG= IFN-γ, TNF= Tumor necrosis factor.

2.12 Deoxyribonucleic acid (DNA) and Riboxynucleic acid (RNA) methodologies

2.12.1 RNA extraction from cells

RNA extraction for gene expression quantification was carried out using the RNeasy kit from Qiagen (Qiagen, West Sussex, UK). Up to 10^7 $CD4^+$ T cells were harvested and washed twice in PBS. Cell pellet was disrupted by adding 350 µl of buffer RLT. The lysate was then pipetted into a QIAshredder spin column plated in a 2 ml collection tube and centrifuged 2 min at full speed. An equal volume of 70% Ethanol was then added to the lysate and mixed. The lysate solution of 700 µl was then transferred into and RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 sec at 12.000 RPM. The flow-through was discarded and 700 µl of

buffer RW1 were added to the RNeasy spin column and centrifuged for 15 sec at 10.000 g. After discarding the flow through, 500 µl of buffer RPE were added to each spin column which was then centrifuged for 2 min at 12.000 RPM to wash the spin column. The RNAeasy spin column was then placed in a new 1.5 ml collection tube and 30-50 µl of RNase free water were added to the spin column which was then centrifuged for 1 min at 12.000 RPM.

2.12.2 RNA quantification

Quality screen and quantification of RNA was performed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). RNA purity and integrity was screened by analysing the ratio of absorbance A260/280 with a value equal or great than 2 indicating high purity of RNA. RNA was stored at -40°C.

2.12.3 Chromatin-immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is a technique used to assess the association of proteins of interest with specific regions of the genome. Cells are fixed *via* formaldehyde treatment and the chromatin is sheared and immunoprecipitated via a highly specific antibody to sequence-specific DNA-binding proteins. To evaluate the binding of ICER/CREM to the IL-2 promoter the MAGnify Chromatin Immunoprecipitation system was used (Invitrogen, Paisley, U.K.). In the first step of the kit Dynabeads were coupled with the antibody of interest. After adding 100 µl of dilution buffer to 10 µl of Dynabeads Protein A/G, the 0.2 ml PCR tubes were placed in the DynaMAg-PCR magnet for 1 min; with the tubes in the magnet the liquid was then discarded without disturbing the bead pellet. After adding 100 µl of the dilution buffer, the antibody of interest (1 µg/sample) and the negative control antibody (provided) were added to the different samples and incubated for 1 hour at 4°C. In order to cross-link DNA and proteins with formaldehyde to preserve the chromatin structure, sorted $CD4^+$ T cells were washed with 10 ml of room temperature PBS. Cells (10^5 per each population analysed) were then resuspended in 500 µl of PBS and 13.5 µl of 37% formaldehyde were added to achieve a final concentration of 1%.

Tubes were mixed by inverting, and incubated for 10 minutes at room temperature. In order to stop the reaction, 57 µl of room-temperature 1.25 M glycine were added to each sample. Samples were mixed by inverting, and incubated for 5 min at room temperature. Cross-linked cells were spun in a cold centrifuge (4°C) at 1200 RPM for 10 min. All tubes were kept on ice from this point. The supernatant was aspirated without disturbing the pellet and cells were gently resuspended in 500 µl of cold PBS. Cells were then centrifuged at 1200RPM for 10 min at 4°C to obtain a pellet which was then wash once more in 500 µl cold PBS.

The chromatin was sheared into 200–500-bp fragments for analysis by quantitative real-time PCR (qPCR) by sonication. Cells were sonicated with 3 cycles of 20 sec. Once the antibody of interest and the negative control antibody have been linked to the Dynabeads, the tubes were spun and placed in the DynaMag PCR Magnet. After discarding the supernatant, the tubes were removed from the magnet and 100 µl of diluted chromatin extract was added to each tube containing the appropriate Antibody-Dynabeads complex. The tubes were rotated end-over-end at 4°C for overnight. The chromatin was then washed using buffer 1 and buffer 2 according to the manufacturer's protocol. The crosslinking was the reversed by placing the tubes washed with IP buffer 2 in the DynaMag-PCR magnet and by adding 54 µl of Reverse Crosslinking Buffer prepared with Proteinase K to each tube. The immune-precipitated (IP) samples were placed at 55°C for 15 min in a thermal cycler. IP sample tubes were placed in the DynaMag™-PCR Magnet and the liquid (containing the sample) was transferred to new labelled 0.2 ml PCR tubes which were then incubated at 65°C for 15 min. The DNA was purified by adding 70 µl of DNA Purification Magnetic Beads prepared with DNA Purification Buffer to each tube. The samples were then incubated at room temperature for 5 min and placed in the magnet in order to remove and discard the liquid. The tubes were removed from the magnet and 150 µL of DNA Wash Buffer were added to each tube. Samples were gently mixed 5 times and the DNA was then eluted by removal of the washed tubes from the magnet and by addition of 150 µL of DNA Elution Buffer to each tube. Samples were incubated at 55°C for 20 min in a thermal cycler, and placed in the magnet for a pellet to form. The liquid which contains the purified samples were then transferred to new tubes and the purified DNA was stored at –20°C.

2.12.4 Polymerase Chain Reaction (PCR)

The DNA immune-precipitated in the Chip study was amplified in a PCR reaction in order to evaluate ICER/CREM binding to IL-2 promoter sequences. The IL2 promoter–specific primer pair 5′-TTACAAGAATCCCAAACT-3′ (forward) and 5′-TAGAGGCTTCATTATCAAA-3′ (reverse), between positions +301 and +510 relative to the promoter site, was used for PCR amplification following the thermo cycles set in the table below.

Table 2.3 PCR reaction cycles

PCR reaction	**t**	**T**
Initial PCR activation step	15 min	95°C
3 step cycle		
Denaturation	1 min	94°C
Annealing	1 min	56-64°C
Extension	1 min	72°C
Number of cycles	25-30	-
Final extension	10 min	72°C

2.12.5 Quantitative real-time (qPCR)

For the quantification of GATA-3 and AEP expression, mRNA was isolated from T cells as described above and equal amounts (250 ng) were reverse-transcribed using the iScript kit (Bio-Rad, Hemel Hempstead, U.K.), generating a master mix for the reactions as in table 2.4. The reverse transcription reaction was carried out by incubating the samples at 25°C for 5 min, followed by a step of 30 min at 42°C and a final incubation of 5 min at 85°C. The cDNA was used in Quantitative real-time PCR reactions containing TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, U.K) (Table 2.5) and a GATA-3-specific primer pair: 5′ CTCATTAAGCCCAAGCGA-3′ (forward) and 5′-TCTGACAGTTCGCACAGGAC-3′ (reverse) used in conjunction with the Universal Probe (UPL) number 8. The primer pair for AEP, 5′ GAACACCAATGATCTGGAGGA-3′ (forward) and 5′-GGAGACGATCTTACGCACTGA-3′ (reverse) was used in conjunction with the UPL probe number 27. The UPL probes are labelled with a fluorescent reporter dye (i.e. FAM or VIC) covalently bound to its 5'-end and a dark quencher dye at the 3'-end. During the PCR reaction, the polymerase cleaves the reporter dye from the probe, releasing it from its quencher and allowing it to fluoresce. The specific hybridization of the target sequence with the specific set of primers and the UPL probe allows the discrimination of the gene of interest. Since UPL probes do not bind double stranded DNA (including primer dimers), the fluorescent dye release corresponds to amplification of the specific target sequence. The probe for the endogenous control 18S has a VIC reporter dye while the GATA-3 and AEP probes have a FAM reporter dye. Probes were used in separate reactions and for each set of primers a water control was performed to control for contamination. The reaction was performed in an ABI Prism 7700 Sequence Detection System with cycles described in table 2.6. Results of individual samples were normalized to that of human 18S rRNA (housekeeping gene; hs9999990_s1, Applied Biosystems). The data obtained from real-time PCR reactions is expressed as arbitrary fold change units and calculated through the Delta-Delta Ct method by normalising the data through subtraction of the Ct value of the endogenous control from the target gene Ct (ΔCt). The ΔCt of the reference or control sample was next subtracted from the ΔCt of the other samples to give the ΔΔCt. Subsequently, the relative quantity of target genes mRNA expression was calculated using the equation: 2^-(ΔΔCt). The ΔΔCt method assumes that standard and target genes have similar

amplification efficiencies. Hence, the validity of the approach should have been confirmed by performing serial dilutions of cDNA template with 18S or target primers/probes to calculate the linear regression slope of the dilution series. This would have allowed to demonstrate that amplification efficiencies of both 18S and target primers/probes were similar (i.e. having slopes with values ranging between -3.60 to -3.10 and an R^2 value of or greater than 0.98).

Table 2.4 Reverse transcription reaction mix

Component	Volume per sample
5x iScript Reaction Mix	4 µl
iScript Reverse Transcriptase	1 µl
Nuclease-free water	Variable
RNA template	Variable
Total Volume	20 µl

Table 2.5 qPCR reaction mix

Component	Volume per sample	Final concentration
TaqMan Universal PCR Master Mix (2X)	10	1X
Forward primer (20X)	1 µl	1X
Reverse primer (20X)	1 µl	1X
TaqMan probe (2.5 µM)	10 µl	250 nM
DNA sample	Variable	10 to 100 ng
Water	Variable	-
Total	20 µl	-

Table 2.6 Thermal cycling conditions

Step	**Hold**	**Cycle (n=35-40)**	
		Deanaturing cycle	**Annealing/Extension cycle**
Temperature	95	95	60
Time	10 min	15sec	1 min

2.13 Wound healing assay

Caco-2 cells were cultured in 24 wells provided by the CytoSelect™ 24-Well Wound Healing Assay Kit. At day 7 of culture, the linear spacer inserted in the well was removed, which created a regular and defined 'wound' within the cell monolayer. Wells were either left untreated or treated with 10 μg/ml mAb to CD46 or an isotype control mAb (conditions were performed in triplicate) and further cultured. Closure of wound areas was monitored by time-laps microscopy (Canon EOS 50D) (Canon UK Ltd., Surrey) and digital pictures were analyzed using MetaMorph software (Molecular Devices, Downingtown, PA).

2.14 Gene array generation

For the methodology of generation of the gene arrays refer to Chapter 4, paragraph 4.3.

2.15 Statistical analysis

Data were expressed as mean ± standard deviation (SD) as indicated in legends. Data were assessed for normal distribution using the D'Agostino-Pearson and the Kolmogorov-Smirnov tests. Statistical analyses were performed using the paired Student's t test (Excel software; Microsoft) when comparing 2 means of normally distributed data. For more than 2 conditions the one way ANOVA was used (GraphPad Prism 4). The Wilcoxon test (2 conditions) or the Friedman test (>2 conditions) were used for non-normally distributed data (GraphPad Prism 4). Unpaired data were tested using unpaired t test for normally distributed data or the Mann-Whitney U test for non-normally distributed data. p values < 0.05 were considered statistically significant.

2.16 Buffers and reagents

All PCR related materials and reagents were purchased from Qiagen, or otherwise specified.

Phosphate buffered saline (PBS): PBS 1X with 0.0067M PO_4 and without Calcium, Magnesium or Phenol Red was purchased from Hyclone, Thermo Scientific (SH30256.021).

Bead buffer and secretion assay buffer: Bovine serum albumin (BSA; Sigma, St. Louis, Missouri) was dissolved in phosphate buffered saline pH 7.2 (PBS) at a final concentration of 0.5% (e.g. 2.5g in 500 ml of PBS). EDTA (EDTA solution pH 8.0 0.5M; Fisher scientific, Loughborough, UK) was added to obtain a 2mM final concentration (e.g. 1.5 ml of 0.5 mM stock in 500ml of PBS). The buffer was stored at 4°C.

Tris-Borate-EDTA: TBE was used for agarose gel electrophoresis and was prepared by adding 108g Tris base, 55g Boric acid and 9.3g EDTA to 1 L of distilled H_2O.

Agarose: Agarose was purchased from Sigma-Aldrich and added to TBE 1X to generate gels for DNA electrophoresis.

Lysis buffer: 1% v/v maltoside, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 10% glycerol.

10X Tris saline buffer-HCl (TBS/Tris-HCl): TBS/Tris-HCl was prepared by dissolving 86.7 g, of sodium chloride (NaCl) and 12.1 g of Tris powder in 900 mL of

dH_2O. The pH was adjusted to 7.4-7.5 using HCl and brought to a volume of 1 L with H_2O. The buffer was stored at room temperature.

TBS TWEEN (TBST): TBS 10X was diluted 10 times in dH2O and 0.05% of Tween 20X (Calbiochem, Nottingham, UK) were added per litre of solution. The buffer was stored room temperature.

Blocking buffer: Powder milk was added to TBST 1X to a final concentration of 5% (e.g 2.5 gr of powder milk in 50 ml of TBST 1X)**.**

PCR Buffer: 10X concentrated buffer contains Tris·Cl, KCl, (NH4)2SO4, 15 mM MgCl2; pH 8.7 (20°C).

CoralLoad PCR Buffer: 10x concentrated buffer contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, gel loading reagent, orange dye, red dye; pH 8.7 (20°C).

$MgCl_2$ solution: 25 mM of $MgCl_2$.

HotStarTaq Plus Master Mix: 2x concentrated master mix contains HotStarTaq Plus DNA Polymerase, PCR Buffer (with 3 mM $MgCl_2$), and 400 µM each dNTP.

DNA size marker: FastRule Low Range DNA Ladder (Fermentas).

Storage and Loading Buffer: 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.005% bromophenol blue, 10% glycerol (Fermentas).

6X MassRuler™ DNA Loading Dye: 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 60% glycerol and 60 mM EDTA (Fermentas).

Tris-Base-EDTA 10X; TBE containing Tris base (890mM), boric acid (890mM) and EDTA (2mM) was purchased from Invitrogen, stored at room temperature and diluted 1:10 before use.

NuPage 2-(N-morpholino) ethane sulfonic acid 20X: NuPage MES was purchased from Invitrogen; 20X buffer contains 50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3.

Protein size marker: SeeBlue Plus2 (Invitrogen) was run on 4–12% Bis-Tris Gel with MES SDS buffer.

Protease inhibitors: Halt Protease inhibitor 100X (Thermoscientific) contained: 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (1mM), Aprotinin 800nM, Bestatin 50μM, (1S,2S)-2-(((S)-1-((4-guanidinobutyl)amino)-4-methyl-1-oxopentan-2yl) carbamoyl)cyclopropanecarboxylic acid, 15μM Leupeptin 20μM, Pepstatin A 10μM.

Chapter 3

CD46-mediated regulation of cytokine secretion in $CD4^+$ human T lymphocytes

3.1 Introduction

The contraction of T cell responses triggered by an infectious pathogen is critical for protection against immunopathologies that arise from exuberant inflammation. Similarly, the prevention of an immune response against self-antigen is vital in the prevention of autoimmunity. The anti-inflammatory cytokine IL-10 plays a key role in limiting immune responses (Fahrer et al., 2001) by inhibiting the production of TNF and IL-12 in macrophages and DCs (Fahrer et al., 2001; Meisel et al., 1996), and by suppressing IL-2 and IFN-γ production by effector T cells (Hahn et al., 2004). The importance of IL-10 for immune homeostasis *in vivo* is highlighted by the fact that IL-$10^{-/-}$ mice succumb to colitis because of their inability to regulate the unwanted immune response against the gut flora (Taub et al., 1993a). Likewise, susceptibility to colitis was recently reported for human families carrying mutations in genes encoding IL-10 receptor chains (Franke et al., 2008). Moreover, while murine IL-10 deficiency accelerates the clearance of infections by *Toxoplasma gondii* or *Trypanosoma cruzi*, the mice rapidly succumb to tissue damage caused by over-production of pro-inflammatory cytokines (Bendelac et al., 1997; Meisel et al., 1996). IL-10 can be produced by many cell types including T and B lymphocytes, DCs, and macrophages (Fahrer et al., 2001). Among T lymphocytes, T_H2 cells and adaptive T-regs have been recognized as prime sources of IL-10 (Benlagha et al., 2000; Fahrer et al., 2001; Meisel et al., 1996). However, it has become increasingly clear that under certain conditions, high amounts of IL-10 may be secreted by some natural T-regs (Inagaki-Ohara et al., 2005), T_H17 cells (Eagle and Trowsdale, 2007), and T_H1 cells (Arase et al., 1994; Meisel et al., 1996; Taub et al., 1993a). This has emphasized the uncertainty regarding the origins of IL-10-producing cells *in vivo*, particularly in humans. IL10-producing T_H1 cells have sparked much interest because they appear key to regulating the immune response to certain infections (Eagle and Trowsdale, 2007; Jankovic et al., 2007; Russell and Ley, 2002), and because their induction may be a mechanism by which tolerance is induced in the presence of persistent (self)-antigen (Hayday, 2009). Thus, there is particular interest in understanding the factors that might regulate IL-10 production by T_H1 cells. In mice, the co-engagement of TCR and CD28 in the presence of high amounts of IL-12 seems to induce strong IL-10 production in T_H1 cells (Saraiva et al., 2009); a similar combination of 'switch receptors' has not been identified yet for human T_H1 cells. However, almost a decade ago the complement

regulatory protein CD46 was identified as one of the strongest inducers of IL-10 in human T cells in *in vitro* cultures: co-engagement of the TCR and the complement regulator CD46 (by antibodies) on human $CD4^+$ T cells, was found to induce a T cell response characterized by high IL-10 secretion, moderate IFN-γ production, granzyme B and perforin expression (Catalfamo and Henkart, 2003; Eagle and Trowsdale, 2007; Inagaki-Ohara et al., 2005). At minimum, CD3/CD46-activated T cells closely resemble Tr1 cells because they produce a similar cytokine pattern (IL-10+, IFN-γ+, IL-4-) and suppress the activation of bystander effector T cells through the immunosuppressive action of IL-10 and independently of Foxp3 (Eagle and Trowsdale, 2007). In addition, both CD46-dependent activation of IL-10 production and Tr1 generation are highly dependent on the presence of exogenous IL-2 (Catalfamo and Henkart, 2003; Caudy et al., 2007; Meisel et al., 1996). Further work showed that also cross-linking of CD46 by its physiological ligands C3b and C4b during TCR activation induces Tr1-like suppressor cells (Shalapour et al.). Likewise, the presence of *Streptococcus pyogenes* (a CD46-binding pathogen) during TCR activation *in vitro* promotes IL-10-secreting Tr1-like cells (Inagaki-Ohara et al., 2005). This suggested that CD46 might be a key factor in IL-10 induction in human $CD4^+$ T cells and that CD46-mediated signals can be induced via its physiological ligands. However, counter-intuitively, although the cell supernatants from TCR/CD46/IL-2 activated T cells were suppressive, they also contained relatively high amounts of the pro-inflammatory cytokine IFN-γ. This was in line with a later study suggesting that CD46 is a strong inducer of a T_H1 phenotype rather than Tr1 cells (Hahn et al., 2004). The key question of my thesis centred on understanding CD46 mediated signals during human $CD4^+$ T cell activation and to assess if and how CD46 regulates possibly both, pro-inflammatory IFN-γ *versus* suppressive IL-10 production in human T cells.

3.2 Hypothesis and Aims

Given the central role of CD46 in the induction of pro-inflammatory (i.e. IFN-γ) and anti-inflammatory (i.e. IL-10) cytokines by human $CD4^+$ lymphocytes it was hypothesised that in human $CD4^+$ T cells the nature of the secreted cytokine (e.g. pro-inflammatory *vs*. anti-inflammatory) was dependent on or could be modulated by the strength of either TCR, CD46 or IL-2 signalling. Hence, the aims of this initial part of the thesis were to identify the significance of IFN-γ production and the characteristics of IL-10 induction by CD46 in human $CD4^+$ T cells. In particular, a mechanism of T_H1-Tr1 "switching" triggered by CD46 was investigated to potentially explain the simultaneous presence of IFN-γ and IL-10 in CD46-activated cultures. Lastly, to further infer the importance of CD46-mediated cytokine production, T cells from patients with T cell-mediated inflammatory disease were analysed *ex vivo*.

3.3 CD46 and IL2 modulate IL-10 secretion of T cells

Activation of purified human $CD4^+$ T cells with stimulating mAbs to CD3 and CD46 in the presence of IL-2 has been shown to induce high IL-10 secretion which mediates a suppressive phenotype, and induce high amounts of IFN-γ typical of T_H1 responses (Sanchez et al., 2004; Shalapour et al.). To replicate these findings human $CD4^+$ T cells were stimulated *in vitro* with plate-bound anti-CD3, anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD46 antibodies in the presence of recombinant human IL-2 (rhIL-2) for 72 hours. Secreted IFN-γ and IL-10 were then quantified by cytometric bead array (CBA) (Figure 3.1). It was demonstrated that upon engagement of CD3 and CD46, greater amounts of IFN-γ and IL-10 were produced in comparison to CD3 or CD3/28 stimulations. To better understand these seemingly paradoxical effects of CD46 on the induction of pro- and anti-inflammatory cytokines, the role of the activating signals was investigated. In particular the CD3, CD46 and IL-2 signals were titrated in order to understand whether the secretion of IFN-γ and IL-10 could be regulated differently and if a particular combination of signals could induce preferential secretion of IL-10 given the previously described regulatory phenotype of CD46-induced $CD4^+$ T cells. Firstly human $CD4^+$ T cells purified from peripheral blood were activated in the presence of a titration of anti-CD3 and anti-CD46

activating antibodies with constant amounts of IL-2 (50 U/ml) (Figure 3.2). It was shown that the levels of secreted IFN-γ were up-regulated in the presence of intermediate (i.e. 1 μg/ml) and high (10 μg/ml) concentrations of either anti-CD3 or anti-CD46 activating antibodies when compared to low strength signalling (i.e. 0.1 μg/ml) except in the case of donor 2 where anti-CD3 and anti-CD46 at 0.1 μg/ml showed high IFN-γ and IL-10 secretion; this finding was not replicated in the other 2 donors (Figure 3.2). Further, IL-10 secretion was found to follow a similar pattern to IFN-γ. However, except from the low strength signal activation in donor 2, none of the activation conditions was able to induce equal amounts of IFN-γ and IL-10 or greater amounts of IL-10 than IFN-γ. Hence, although showing a modulation of cytokine secretion, with a tendency of highest secretion in response to the strongest strength signal, the titration of CD3 and CD46 signals did not induce a 1:1 ration between IFN-γ and none of the activating conditions showed higher IL-10 than IFN-γ (Figure 3.2).

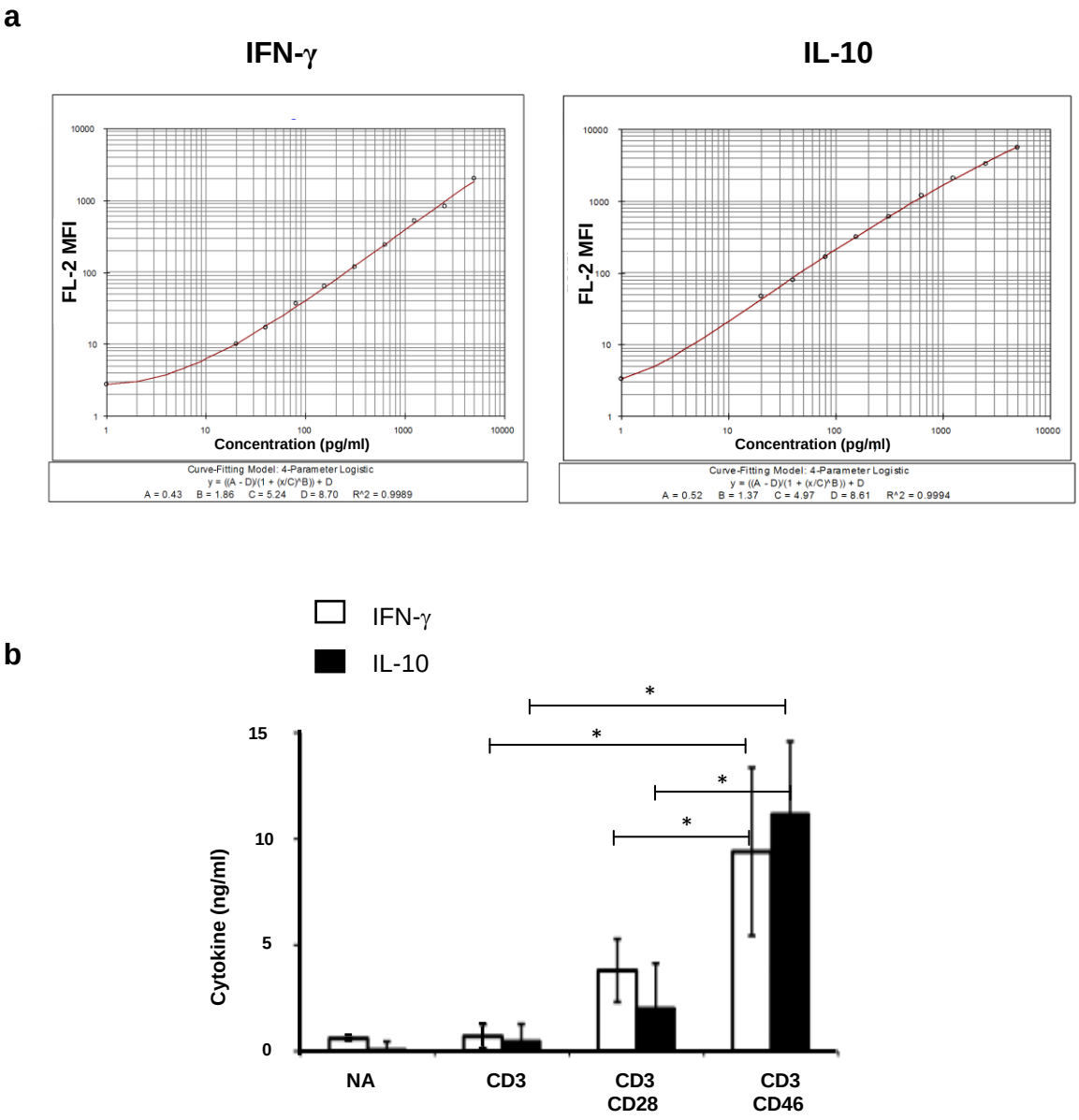


Figure 3.1 CD4+ T cells activated via CD3 and CD46 secrete IFN-γ and IL-10. Purified human CD4+ T cells were activated in a 96 well plate and seeded at a concentration of 1.5 x 10^5 per well. Each activation was performed in triplicate with the indicated stimulating antibodies in the presence of 50 U/ml rhIL-2. At day 3 post activation supernatants were harvested and IFN-γ and IL-10 amounts were measured in the cell supernatants through CBA. a) CBA standard curves for IFN-γ and IL-10 quantification expressed in picograms (pg) per ml. b) IFN-γ and IL-10 amounts in supernatants of non-activated (NA), anti-CD3 (CD3), anti-CD3 plus anti-CD28 (CD3/CD28) or anti-CD3 plus anti-CD46 (CD3/CD46) activated samples. Data represent means ± SD of 8 separate experiments. p-values derive from one-way ANOVA test. *, $p < 0.05$ assessed by Bonferroni paired post-test for multiple comparisons.

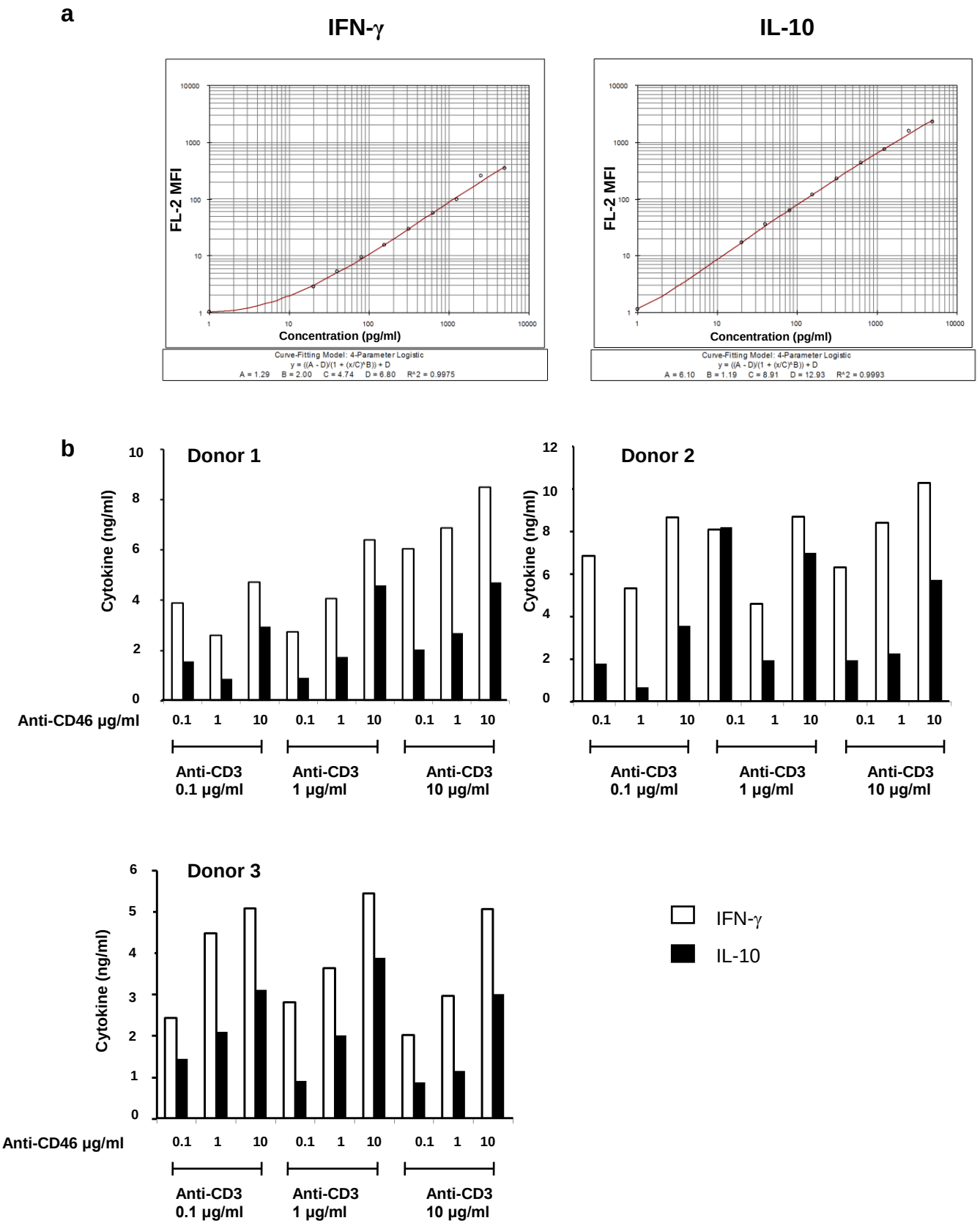


Figure 3.2 CD3 and CD46 signal strength titration in the modulation of cytokine secretion. CD4+ T cells were purified via magnetic cell sorting (positive selection) from the peripheral blood of 3 healthy donors. Purified CD4+ lymphocytes were

seeded in a 96 well plate and activated with plate-bound mAbs in the presence of 50 U/ml of rhIL-2. $CD4^+$ T cells were activated with anti-CD3 mAbs at a concentration of 0.1, 1 or 10 μg/ml and in combination with anti-CD46 mAbs at a concentration of 0.1, 1 or 10 μg/ml as indicated. At day 3 post activation supernatants were harvested and the amounts of IFN-γ and IL-10 were measured for each activation condition through CBA. a) Standard curves for IFN-γ and IL-10 quantification. b) IFN-γ and IL-10 amounts expressed in ng/ml for each of the activation conditions.

Next the IL-2 signals were investigated in order to understand whether added exogenous IL-2 could impact on IFN-γ and IL-10 secretion and favour IL-10 secretion in specific activating conditions. When human $CD4^+$ T cells were activated in the presence of increasing concentrations of IL-2, but with constant anti-CD3 and anti-CD46 strength signal (2 μg/ml), cytokine production of both IFN-γ and IL-10 was also found to be regulated (Figure 3.3). Specifically, in very low (<0.5 U/ml) IL-2 concentration, CD3/CD46 activation induced high IFN-γ secretion, compared to T cells activated with anti-CD3 or anti-CD3 plus anti-CD28. Increasing IL-2 beyond 5 U/ml did not further increase IFN-γ production, but in IL-2 concentrations of 5-10 U/ml, CD3/CD46-activated T cells from different donors all displayed strong IL-10 secretion in addition to IFN-γ, with the level of IL-10 produced increasing with the amount of IL-2. Hence, high IL-2 in combination with anti-CD3 and anti-CD46 activating signals was found to be a condition able to induce high IL-10 secretion and that could favour IL-10 induction more than IFN-γ. The analysis of the kinetic of cytokine secretion showed that CD46-induced IFN-γ peaked 24 hours post activation and then steadily declined, whereas IL-10 was barely detectable before 24 hours and peaked at 72 hours (Figure 3.4). Thus, CD46-mediated IFN-γ production preceded IL-10 secretion even in high IL-2 cultures that are most conducive to IL-10 production. The addition of a neutralizing mAb to IL-2 during CD3/CD46 activation abrogated cell proliferation and cytokine production (Figure 3.4).

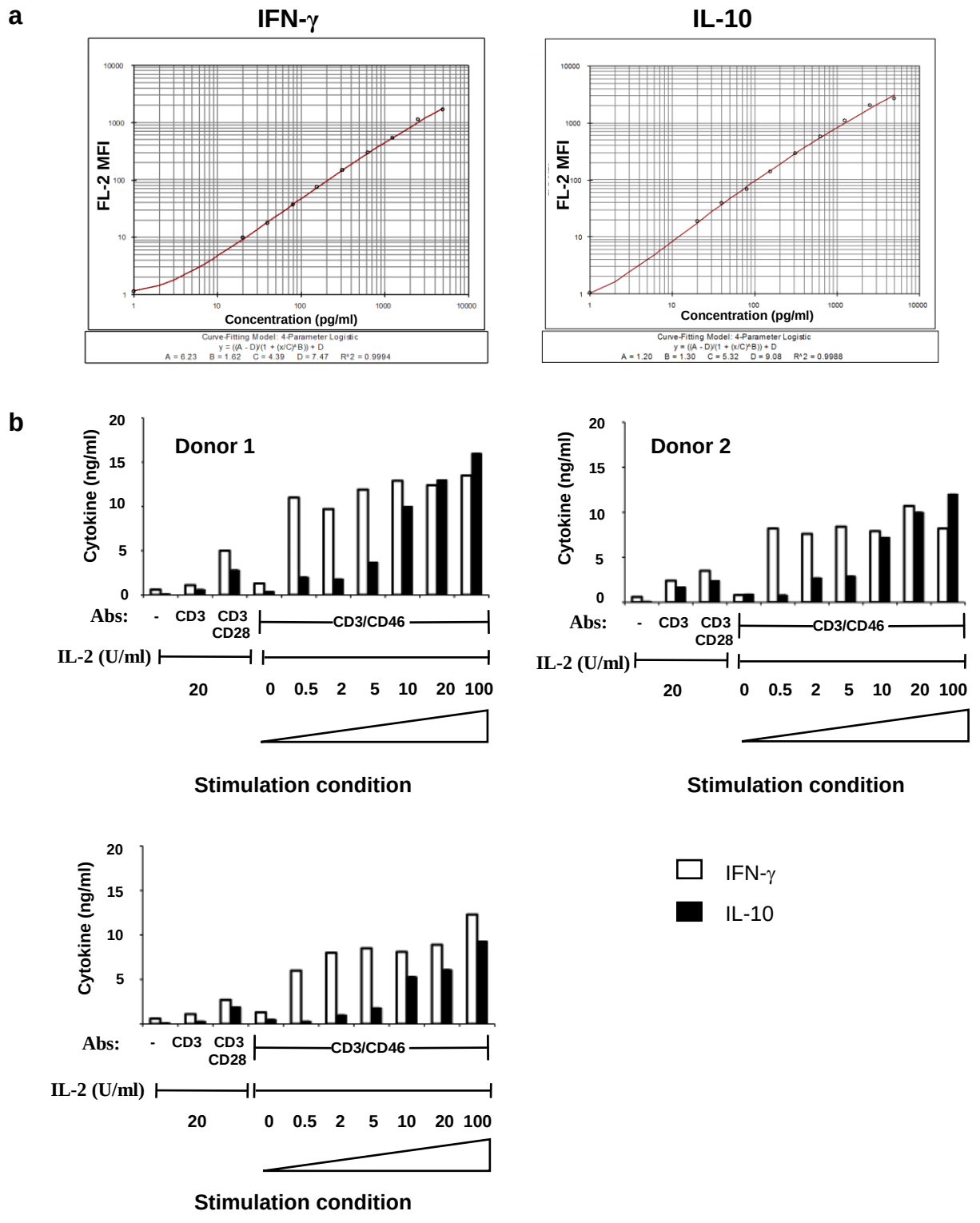


Figure 3.3 CD46 and IL-2 regulate IFN-γ and IL-10 secretion by TCR-activated CD4+ T cells. Purified human CD4+ T cells were purified from 3 healthy donors via magnetic cell sorting (positive selection). T cells were either left non-activated (-) or activated with anti-CD3 (CD3), anti-CD3 plus anti-CD28 (CD3CD28) in the presence of 20 U/ml of rhIL-2 or with anti-CD3 plus anti-CD46 (CD3CD46) mAbs with increasing amounts of rhIL-2. The concentrations of rhIL-2 ranged between 0 and 100 U/ml. At day 3 post activation, IFN-γ and IL-10 amounts were measured in the cell supernatants through CBA. a) CBA standard curves for IFN-γ and IL-10. b) IFN-γ and IL-10 amounts expressed in ng/ml for each of the activation conditions.

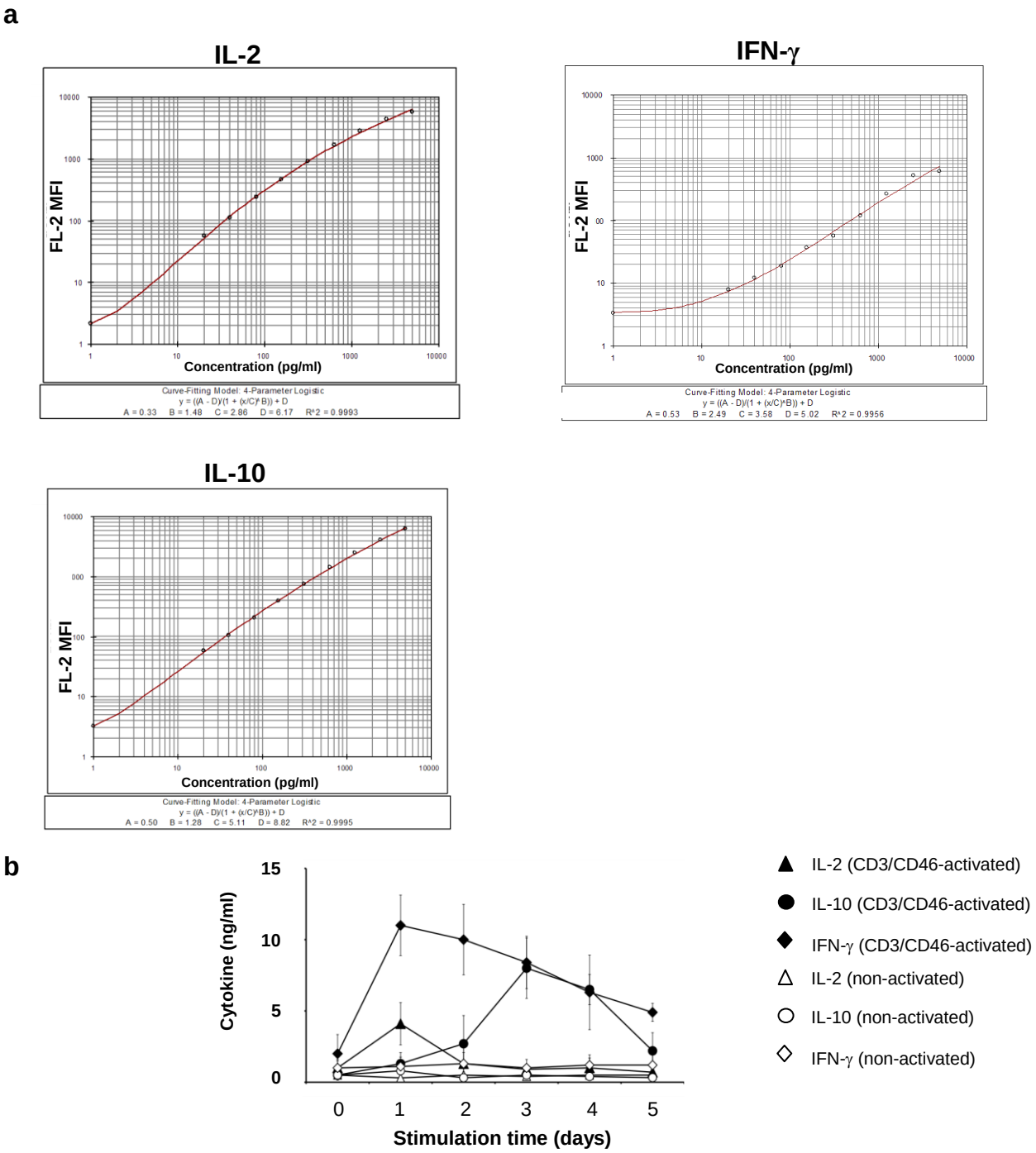


Figure 3.4 Kinetics of IL-2, IL-10 and IFN-γ secretion in CD3/CD46 activated T cells. Purified CD4+ T cells were left non-activated (white shapes) or activated (black shapes) with mAbs to CD3 and CD46 for up to 5 days. At each of the indicated time points supernatants were harvested to determine IFN-γ, IL-2 and IL-10 amounts by CBA. IFN-γ and IL-10 secretion was assessed in cultures containing 50 U/ml rhIL-2, while the experiments measuring IL-2 secretion were performed without the addition of exogenous rhIL-2. a) CBA standard curves for IL-2, IFN-γ and IL-10. b) Cytokine contents of indicated supernatants. Data represent means ± SD of 3 separate experiments.

3.4 Successive induction of IFN- γ (T_H1) and IL-10 (Tr1) cells upon CD46 signals

The single cell dynamics of IFN-γ and IL-10 secretion mediated by the engagement of the TCR and CD46 were next investigated. Purified $CD4^+$ T cells were activated with mAbs to CD3 alone or in combinations with mAbs to CD28 and CD46 in the presence of a neutralizing mAb to IL-2, or either low (5 U/ml) or high (50 U/ml) doses of IL-2. Active cytokine secretion was measured 36 hours post activation (Figure 3.5). As stated above, blockage of IL-2 inhibited cytokine production under any conditions analysed. However, in the presence of IL-2, anti-CD3/CD46 activation and anti-CD3/CD28/CD46 activation each induced three discrete T cell populations with distinct secretion profiles: IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells (Figure 3.5). Cultures containing 5 U/ml IL-2 had higher frequencies of IFN-γ secreting cells (17.5% ± 5.2%, IFN-γ+/IL-10(-) and IFN-γ+/IL-10+) compared to IL-10-producing T cells (12.1% ± 1.8%, IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+), whereas 50 U/ml IL-2 induced a significant increase in the double positive IFN-γ+/IL-10+ (26.3% ± 5.8% IL-10+) and single positive IFN-γ(-)/IL-10+ cells (12.2% ± 3.5% IL-10+). By contrast, although CD3/CD28-stimulated cultures also contained all three IFN-γ/IL-10-secreting T cell populations, there was no IL-2-dependent change in the ratio of IFN-γ producing vs. IL-10-positive T cells, and the total number of IL-10-producing cells remained low even in high IL-2 (Figure 3.5). The ability of the different cell populations induced by anti-CD3/CD46 to regulate T cell activation was then assessed. $CD4^+$ T cells were activated through CD3/CD46 ligation and subsequently sorted into IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cell subsets, which were then cultured for 18 hours in 1 U/ml IL-2. The supernatants of the three cell types continued to contain high IFN-γ, approximately equal amounts of IFN-γ and IL-10; and high amounts of IL-10, respectively (Figure 3.6). When freshly purified $CD4^+$ T cells were cultured in the presence of these supernatants together with cross-linking antibodies to CD3 and CD28, their proliferation, measured at day 6, was substantially inhibited by the supernatants of the CD3/CD46-induced IFN-γ+/IL-10+ cells, and of the IFN-γ(-)/IL-10+ T cells (Figure 3.6). The inhibition was not evoked by supernatants from IFN-γ+/IL-10(-) T cells, nor by supernatants from T cells activated for 72 hours via CD3 and CD28. It was, however, almost completely blocked

by a neutralizing mAb to IL-10, demonstrating that the IL-10 secreted in response to CD3/CD46 activation is the primary soluble mediator of regulation from these cells.

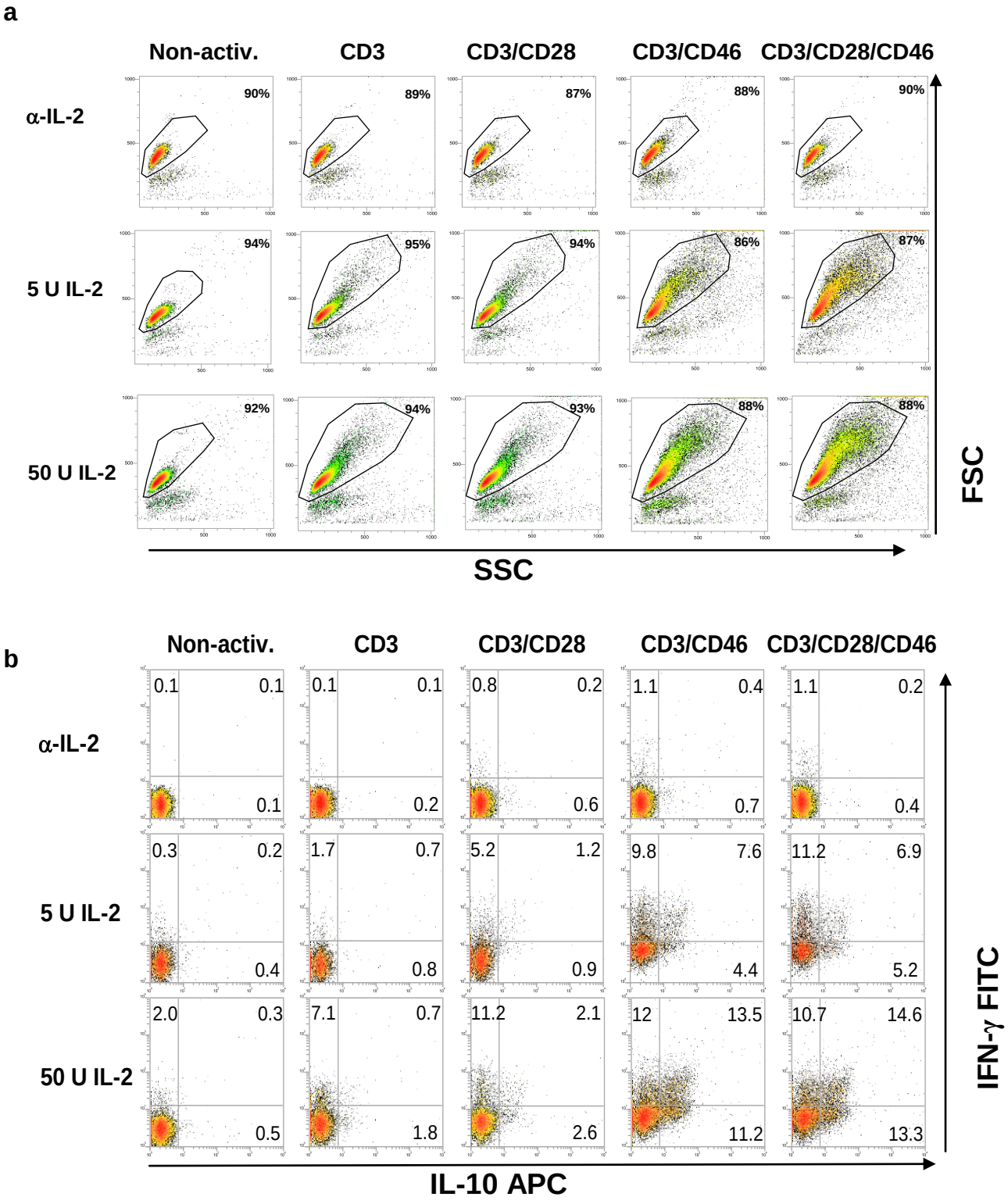


Figure 3.5 CD46 and IL-2 induce three distinct populations of IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells. a) Purified T cells were either left non-activated (Non-activ.) or activated with plate-bound mAbs to CD3, CD3 plus CD28

(CD3/CD28), CD3 plus CD46 (CD3/CD46) or CD3 plus CD28 plus CD46 (CD3/28/46) at a concentration of 2 ug/ml for each of the mAbs. Stimulations were carried out in the presence of a function-neutralizing mAb to IL-2 or with the addition of 5 or 50 U/ml rhIL-2. Following 36 hours of activation cells were harvested and active IFN-γ and IL-10 secretion was assessed using a combined cytokine secretion assay specific for IFN-γ and IL-10. a) FSC/SSC gating strategy for the blasting activated T cells. b) Percentages of IFN-γ and IL-10 secreting T cells in the indicated activating conditions. Values represent the percentage of positive cells in the relevant quadrant. Shown are the results from one representative experiment of 6 performed separately.

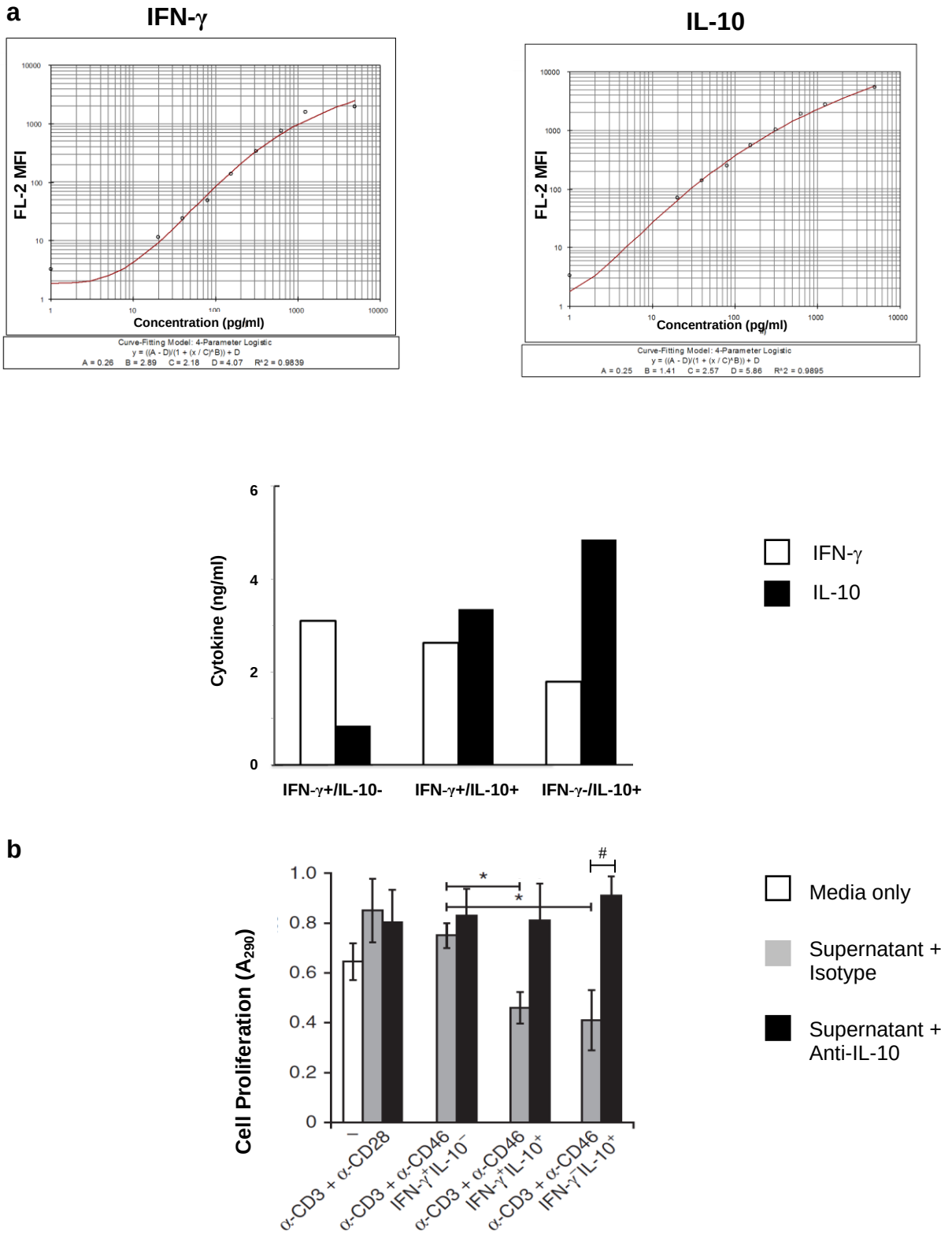


Figure 3.6 Suppressive abilities of the three distinct populations of IFN-γ+IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells induced by CD46 and IL-2. a) Purified CD4+ T cells were activated with mAbs to CD3 and CD46 (2 μg/ml) in the presence of 50 U/ml of rhIL-2. The resulting IFN-γ+IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+

populations were FACS-sorted according to the cytokine secretion profiles and rested for 18 hours. a) Supernatants from IFN-γ+/IL-10(-), IFN-γ+/IL-10+, IFN-γ(-)/IL-10+ cultures at 18 hours were harvested and quantified for secreted IFN-γ and IL-10 by CBA. Top panels represent the CBA standard curves for IFN-γ and IL-10. b) Purified $CD4^+$ T cells were activated with mAbs to CD3 and CD28 and cultured in the presence of media only (white bar), with the supernatant derived from anti-CD3 plus anti-CD28 (αCD3+αCD28) activated T cells or with supernatants from the sorted IFN-γ+/IL-10+, IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ populations in the presence of an isotype (IgG1, 10 μg/ml) control antibody (grey bars). Black bars indicate the addition of a blocking antibody to IL-10 (10 μg/ml) to the indicated supernatants. Proliferation was measured at day 6. Data are representative of three experiments. *$p < 0.05$ derived from Friedman's test with Dunn's multiple comparisons. $^{\#}p < 0.05$ derived from Wilcoxon test.

The described experiments were performed with purified $CD4^+$ T cell populations that at minimum include naïve and memory $CD4^+$ T cells, and natural T-regs. To exclude the possibility that the observed CD46-elicited populations reflect selective reactivation and/or expansion of one or more of these lineages, the anti-CD3/CD46 activation protocols were applied to naïve T cells. In order to obtain highly purified naïve T cells, $CD4^+$ T cells were purified from peripheral blood and subsequently surface stained for CD4, CD45RA, CD45RO, CCR7 and CD25. Naïve T cells were FACS-sorted as $CD4^+/CD45RA^+/CD45RO^-/CCR7^-/CD25^-$ lymphocytes as shown in Figure 2.3. Post sort naïve T cells were activated with plate-bound antibodies to CD3 and CD46 for 36 hours in the presence of 50 U/ml IL-2 (Figure 3.7). Subsequently, Dr Kemper and Dr Le Frïec analysed the secretion profiles for IFN-γ and IL-10 upon 3 successive rounds of stimulation with either CD3 mAbs or CD3 and CD46 mAbs. The data generated showed that IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells were induced in low frequencies during the 1^{st} round of stimulation (Figure 3.7). The use of anti-CD3/CD46 to re-stimulate naïve cells previously activated and expanded with mAbs to CD3 and CD46 substantially increased the proportion of both IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ T cells (Figure 3.7). Moreover, each additional CD3/CD46 re-stimulation evidently increased the frequencies of IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells compared to the previous activation cycle.

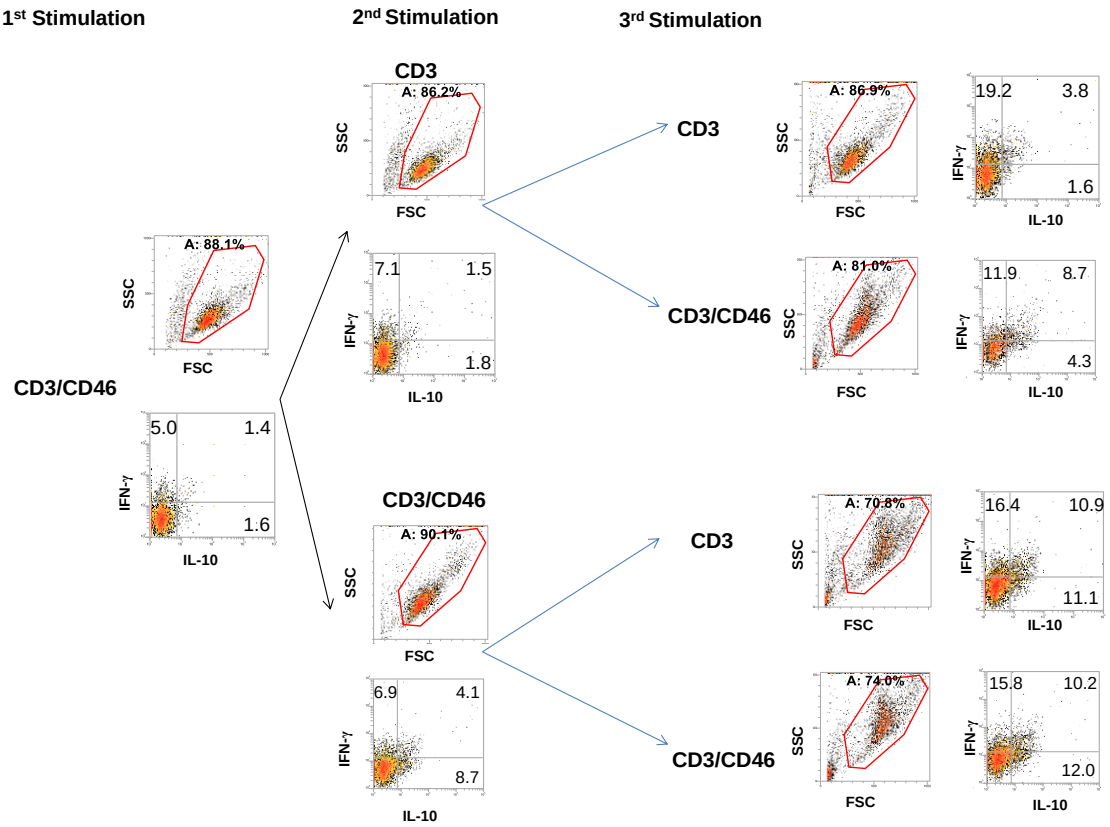


Figure 3.7 Naïve CD4$^+$ T cell cultures gradually acquire the 'phenotype' of CD3/CD46-activated T cells cultures. Naïve human CD4$^+$ T cells (CD4$^+$/CD45RA$^+$/CD45RO$^-$/CD25$^-$/CCR7$^+$) were isolated through FACS-sorting as described in figure 2.3 and activated with mAbs to CD3 and CD46 in the presence of 50 U/ml rhIL-2 for 36 hours (1st stimulation). Cells were expanded 5 days in the presence of 5 U/ml rhIL-2 and then restimulated (2nd stimulation) with mAbs to either CD3 or to CD3 plus CD46. Cells were then rested and re-stimulated for a 3rd time (3rd stimulation) as shown in the presence of 50 U/ml rhIL-2. After each round of activation IFN-γ and IL-10 secretion was measured by cytokine secretion assay. Data shown are representative of two similarly performed experiments. ^Data provided by Dr Le Frïec and Dr Kemper.

3.5 CD46-induced IL10 production originates in a T_H1 subset

In accordance with the last data sets, several publications suggested that the major source of IL-10 *in vivo* may be T_H1 cells that begun co-producing this cytokine in addition to IFN-γ (Meiler et al., 2008) rather than a designated lineage such as Tr1 cells. Thus, in the next set of experiments, the origin of CD46-induced IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ T cells was investigated. As diagrammed in Figure 3.8 two hypotheses were viable: 1) IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ T cells could generate from the IFN-γ+/IL-10(-) T_H1 cells or 2) Each cell population could arise from separate cell populations. Two sets of experiments were performed. First, purified $CD4^+$ T cells were activated via CD3 and CD46 in high IL-2 in the presence of neutralizing mAbs to IFN-γ, a condition known to inhibit T_H1 development (Murphy et al., 2000). Subsequently Dr Kemper performed secretion assays in order to identify IFN-γ and IL-10 secreting T cells in the different conditions. A significant decrease in the number of IFN-γ+/IL-10(-) cells was observed after 48 hours (from 14% ± 4% to 4% ±2.5%, Figure 3.8). However, this treatment also substantially reduced the percentages of CD46-induced IFN-γ+/IL-10+ cells from 16% ± 2% to 2.5% ± 1.5%, and of the IFN-γ(-)/IL-10+ cells from 18% ± 3% to 1.5% ± 0.5%. Thus, limiting T_H1 differentiation limits the "IL-10-switch". IL-10 neutralization did not decrease the cell numbers of any of the T cell populations induced but rather slightly increased their representation (IFN-γ+/IL-10(-) from 14% ± 4% to 17.5 ± 4%; IFN-γ+/IL-10+ from 16% ± 2% to 19.5% ± 6.5%; IFN-γ(-)/IL-10+ from 18% ± 3% to 18.5% ± 2.5%, Figure 3.8). These data suggest at minimum that the continuous production of IL-10 initially induced via CD3/CD46/IL-2-mediated signals does not depend on positive feedback by IL-10 itself. Second, purified $CD4^+$ T cells were activated with plate bound mAbs to CD3 or CD3/CD46 in the presence of low concentrations of rhIL-2 (5 U/ml). The resulting IFN-γ+/IL-10(-) cells were isolated via FACS sorting in collaboration with Dr Le Frïec. When, after 4 days of expansion, the cells were re-stimulated by Dr Le Frïec via CD3/CD46, they produced a high percentage of IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells (Figure 3.9). Taken together, these two data sets strongly suggest that CD46-induced IL-10-secreting T cells derive from an initial T_H1 phase. In fact, whilst the inhibition of IFN-γ through a blocking antibody showed that the induction of IFN-γ production by CD46-activated T cells, and consequently the signalling of this cytokine, is a requirement for the generation of IFN-γ+/IL-10(-),

IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells, these set of experiments were not definitive in determining whether IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells derived from the IFN-γ+/IL-10(-) population. However, the second set of experiments, where IFN-γ+/IL-10(-) cells were isolated through FACS sorting and restimulated through CD3 and CD46 were indicative of the ability of purified CD46-induced IFN-γ+/IL-10(-) T cells to switch towards the production of IL-10. Furthermore, the importance of CD46 signalling in these experiments was highlighted by the weaker switch of IFN-γ+/IL-10(-) when the restimulation condition only included mAbs to CD3.

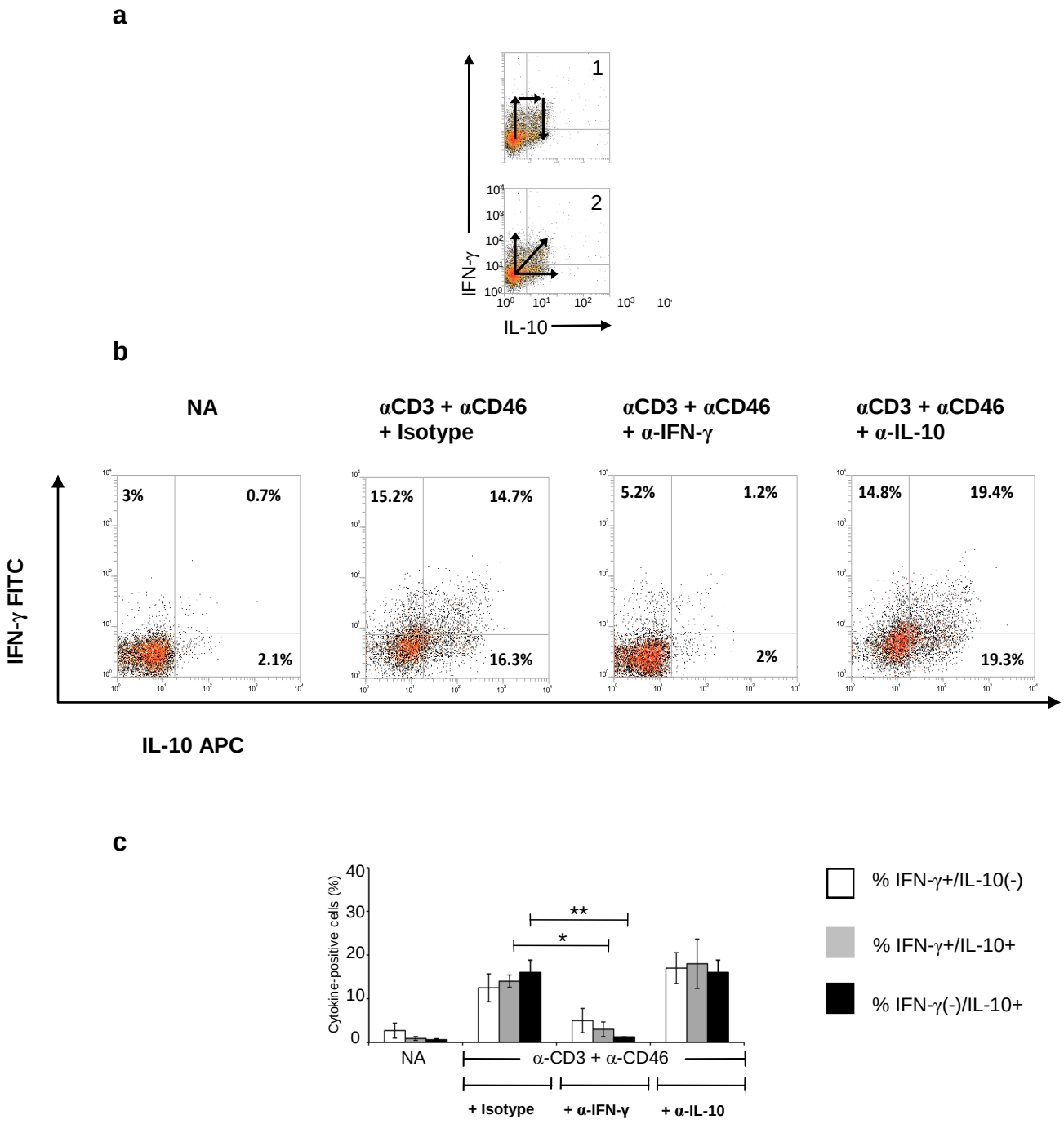


Figure 3.8 Anti-IFN-γ treatment inhibits the generation of CD46/IL-2 induced IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ populations. (a) CD46-induced suppressive IL-10-secreting cells could originate via an initial T_H1 'phase' (1) or arise from a separate cell population (2). (b) $CD4^+$ T cells were purified via magnetic cell sorting and were either left non-activated (NA) or activated with plate bound mAbs to CD3 and CD46 (αCD3 + αCD46) in the presence of 50 U/ml of rhIL-2. Anti-CD3 plus anti-CD46 activated T cells were activated in the presence of an isotype (IgG1, 10 μg/ml) control antibody or in the presence of function-neutralizing mAbs to either IFN-γ (10 μg/ml) or IL-10 (10 μg/ml). After 48 hours of activation cytokine secretion was assessed using the cytokine secretion assay. Values shown are indicative of the percentage of positive cells within the relevant quadrant. c)

Percentages of cytokine-positive cells (IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+) under each activation condition. Data shown represent mean +/- SD of three separately performed experiments with activation conditions analysed in duplicates. *p < 0.05 derived from Friedman's test with Dunn's multiple comparisons. ^, Data generated in collaboration with Dr Kemper.

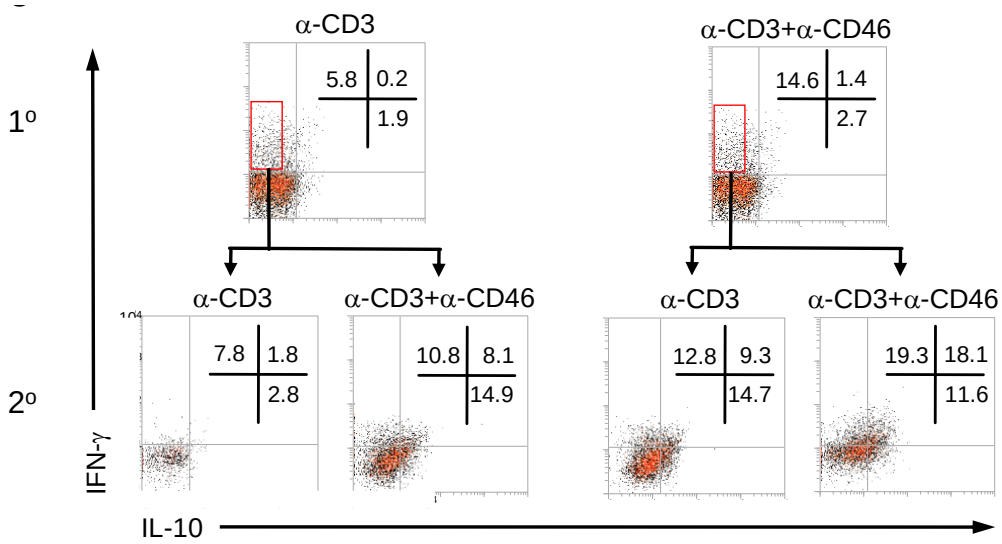


Figure 3.9 CD46/IL-2 signals induce a switch from a T_H1 into a suppressive phenotype in CD4+ T cells. CD4+ purified T cells were activated with plate bound mAbs to CD3 or CD3/CD46 each at 2 μg/ml for 36 hours in the presence of 5 U/ml of rhIL-2. Cells were stained through a secretion assay for IFN-γ(FITC) and IL-10(APC) secretion, FACS-sorted according to the gates shown and expanded for 4 days in 5 U/ml IL-2. Cells were then restimulated with mAbs to CD3 (α-CD3) or CD3 plus CD46 (α-CD3+ α-CD46) and IFN-γ (FITC) and IL-10 (APC) secretion was assessed 18 hours post activation through a secretion assay. Data shown are representative of two similarly performed separate experiments. ^Data in this figure were generated in collaboration with Dr Le Frïec.

3.6 CD3/CD46-induced IL-10 cells retain T_H1 markers

T cell lineages are characterized by specific markers: T_H1 differentiation is accompanied by signal transducer of activation (STAT)1 and STAT4 phosphorylation and T-box 21 (T-bet) expression; T_H2 cells require STAT6 activation and expression of GATA-binding protein (GATA) 3 (Catalfamo and Henkart, 2003; Glimcher and Murphy, 2000); natural T-regs are characterized by forkhead box P3 (FoxP3) expression (Lewis et al., 2006). When purified $CD4^+$ T cells were activated *via* CD3/CD46 for 36 hours in high IL-2, the resultant IFN-γ+/IL-10(-), and IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells expressed predominantly a T_H1 profile (Figure 3.10 and Table 3.1), as reflected by strong T-bet expression and STAT4 phosphorylation in conjunction with low GATA3 activation and STAT6 phosphorylation. In the mouse, maintenance of IL-10-producing T_H1 cells requires sustained expression of pSTAT4, T-bet, the extracellular-signal regulated kinase (ERK), and the mitogen-activated protein kinase (MAPK) (Gabrysova et al., 2009; Saraiva et al., 2009). Likewise, CD46-dependent, TCR-independent induction of ERK1/2 in human $CD4^+$ T cells was previously reported (Zaffran et al., 2001). It was confirmed that all three CD46-induced T cell populations contained high amounts of pERK1/2 (Table 3.1). Signalling through the IL-2 receptor complex induces Janus kinase (JAK)-mediated STAT5 phosphorylation. Consistent with the fact that CD46-induced IL-10 production is IL-2-dependent, high expression of pSTAT5 was detected in IFN-γ+/IL-10(-), and IFN-γ+/IL-10+ cells, although it was lower in IFN-γ(-)/IL-10+ T cells, perhaps consistent with their limited proliferative capacity (Catalfamo and Henkart, 2003). In summary, the data generated and shown in Figures 3.1 to 3.10 support the notion that the IL-10-producing T cells originate via an initial IFN-γ-producing Th1 ‘phase’.

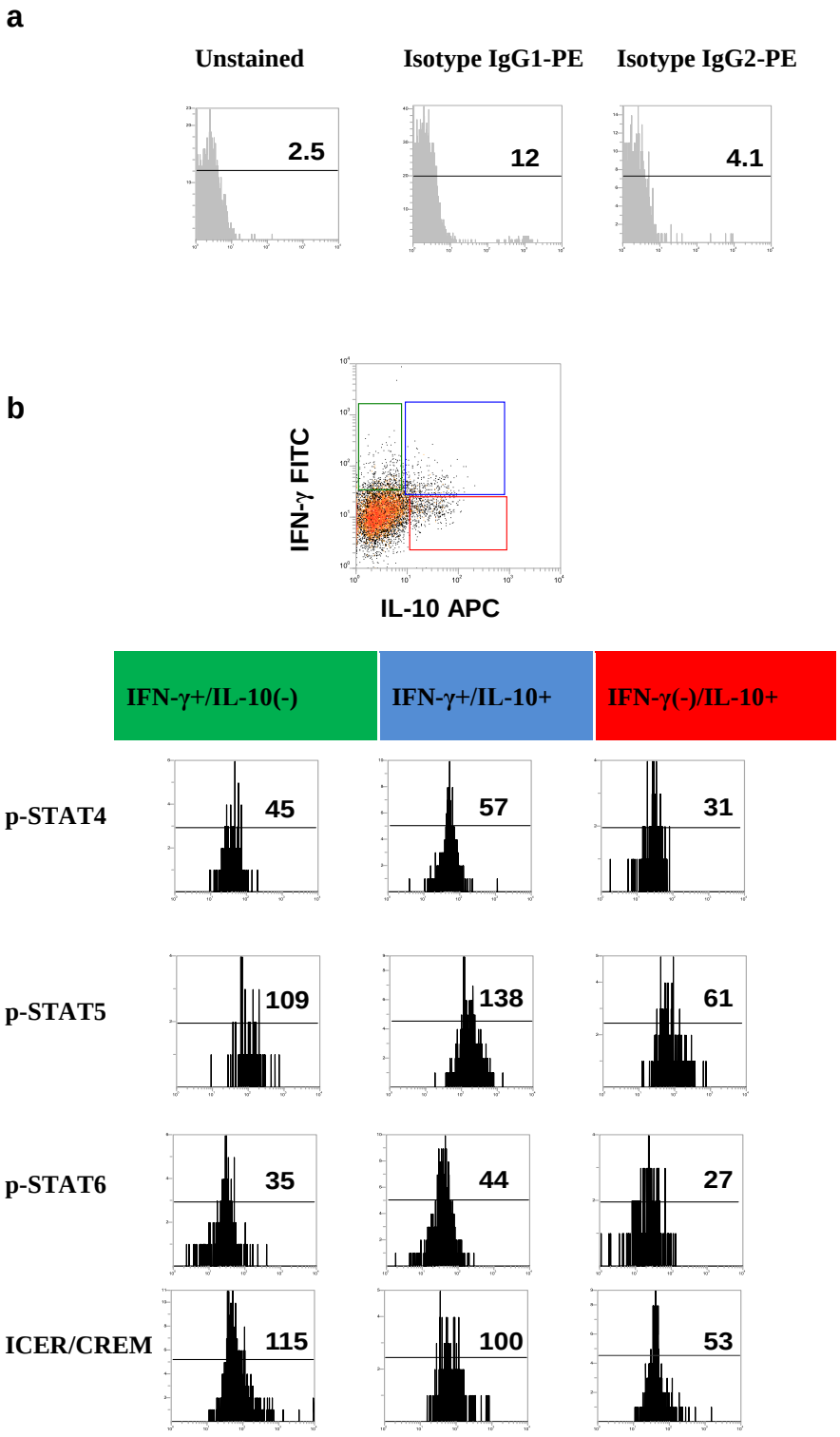


Figure 3.10 p-STAT4, p-STAT5, p-STAT6 and ICER/CREM expression in the CD46-induced IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ populations. $CD4^+$ T cells were purified via magnetic isolation (positive selection) and activated with plate-bound mAbs to CD3 plus CD46 (2 μg/ml) in the presence of 50 U/ml of rhIL2. After 36 hours of activation a secretion assay for IFN-γ (FITC) and IL-10 (APC) was performed to discriminate the IFN-γ+/IL-10(-), and IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ populations. Cells were then fixed/permeabilized and stained

intracellularly for p-STAT4, p-STAT5, p-STAT6 or ICER/CREM. a) Unstained and isotype controls. b) Colour-coded gating strategy for IFN-γ+/IL-10(-) (green gate), IFN-γ+/IL-10+ (blue gate) and IFN-γ(-)/IL-10+ (red gate) populations and relative histograms. Values represent the mean fluorescent intensity (MFI) values for the relative histogram. Data are representative of 2 experiments.

Transcription factor	IFN-γ+/IL-10(-)	IFN-γ+/IL-10+	IFN-γ(-)/IL-10+	T_H1[a]	T_H2[a]
T-bet	+++	+++	+++	++++	-
Gata3[b]	-/+	+	+	-/+	++++
Foxp3	-	-	-	-	-
p-STAT4	++	+++	++	++++	++
p-STAT5	+++	+++	+	++++	++
p-STAT6	+	+	-/+	+	++++
p-ERK	++	+++	+++	++++	++
ICER/CREM	+++	++	+	++	++

Table 3.1 Expression profile of key T cell lineage transcription factors in CD46-induced effector and suppressor cell populations. Purified $CD4^+$ T cells were CD3/CD46-activated for 36 hours in 50 U/ml of rhIL-2 and the subsequent IL-10 and IFN-γ secretion assays combined with intracellular FACS staining for the respective T cell lineage markers. [a], transcription factor expression levels were determined relative to the expression levels in either T_H1 or T_H2 cells with ++++, very strong (highest expression observed); +++, strong; ++, moderate; +, low; -/+, weak but detectable; -, not detectable. [b], due to the lack of a suitable Ab, mRNA GATA3 levels were determined using quantitative real-time RT-PCR analysis.

3.7 IL-2 regulation by CD3/CD46

An interesting and widely accepted observation is that all IL-10-producing T cells with regulative capacity lose the capacity to coproduce IL-2 (Gabrysova et al., 2009; Saraiva et al., 2009; Meiler et al., 2008). This is particularly true for the IL-10-secreting cells that arise from T_H1 cells. Thus, to draw further parallels with T_H1 cells, IL-2 expression in the three distinct IFN-γ and/or IL-10-secreting cell populations was examined (Gabrysova et al., 2009; Hayday, 2009). CD46-dependent IL-2 expression was detected in CD3/CD46-activated $CD4^+$ T cell cultures (Figure 3.11). More than 85% of IFN-γ+ cells actively secreted IL-2 at 36 hours post CD3/CD46 activation (Figure 3.11). However, less than 25% of IL-10-secreting cells were co-secreting IL-2. Thus, CD3/CD46-activated cells shared with T_H1 cells the production of IL-2, which ceased with the induction of the IL-10-secreting state. Gene array data generated by Dr. Claudia Kemper previous to the start of the project were used to identify possible regulators of IL-2 transcription. The gene array was based on the comparison of CD3/CD46-activated versus CD3/CD28-activated primary $CD4^+$ T cells and revealed significantly higher levels of mRNA encoding the ICER/CREM protein in CD46-activated cells. Translocation to the nucleus of ICER/CREM causes transcriptional attenuation of IL-2 (Powell e al., 1999). When $CD4^+$ T cells were activated through CD3 plus CD46 mAbs it was confirmed that CD3/CD46 ligation induced up-regulation of ICER/CREM protein expression (Figure 3.12). The level of ICER CREM expression was also assessed by FACS on the three distinct populations induced by CD46 and IL-2 (i.e IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+) (Figure 3.10). Unexpectedly, ICER/CREM expression was highest in IFN-γ+/IL-10(-) cells, which continue to express IL-2 (Figure 3.10). However, analysis by western blot of cytoplasmic and nuclear fractions revealed that ICER/CREM nuclear translocation was only consistently detected 48 hours post CD3/CD46 stimulation (Figure 3.13), which coincides with the appearance of IL-10 in the culture medium (Figure 3.4). Activation of T cells through CD3 and CD3/28 activation did not lead to ICER/CREM nuclear translocation at any time point (Figure 3.13). It is important to notice that the detection of ICER/CREM by flow cytometry (Figure3.12) did not reflect the findings of protein expression obtained by western blot (Figure 3.13). In fact, the staining for ICER/CREM in $CD4^+$ T cells activated with anti-CD3 or anti-CD3 plus anti-CD28 by FACS showed increased ICER/CREM expression in comparison to unstimulated cells.

The same finding was not replicated in the western blot analysis where only activation conditions including CD46 determined ICER/CREM detection. A possible explanation is given by the sensitivity of the different techniques. Flow cytometry has higher sensitivity in the detection of antigens and this may explain the different results obtained. To determine if ICER/CREM bound preferentially to the IL-2 promoter in IL-10+ cells, chromatin Immunoprecipitation (ChIP) using an ICER/CREM-specific mAb was applied to IFN-γ+/IL-10(-) and IFN-γ(-)/IL-10+ cells purified 36 hours post CD3/CD46 activation. IL-2 promoter-specific DNA sequences were precipitated only from IL-10+ cells, and not from IFN-γ+ T cells (Figure 3.13c). Thus, CD3/CD46 activation induces strong ICER/CREM expression in T_H1 cells but this is inactive in IFN-γ+ cells that continue to produce IL-2. Instead, active nuclear translocation occurs preferentially in IL-10+ cells, consistent with their decreased IL-2 production. ChIP was only performed on the IFN-γ+/IL-10(-) and IFN-γ(-)/IL-10+ populations. The IFN-γ+/IL-10+ T cells were not considered in this set of experiments because it was postulated that the comparison between IFN-γ+/IL-10(-) and IFN-γ(-)/IL-10+ cells would have given a neat result in terms of IL-2 promoter binding by ICER/CREM when either IFN-γ or IL-10 were the main cytokine produced. However, the ChIP analysis of IFN-γ+/IL-10+ could have provided further evidence of ICER/CREM translocation by showing an "intermediate" state of IL-2 promoter binding by ICER/CREM.

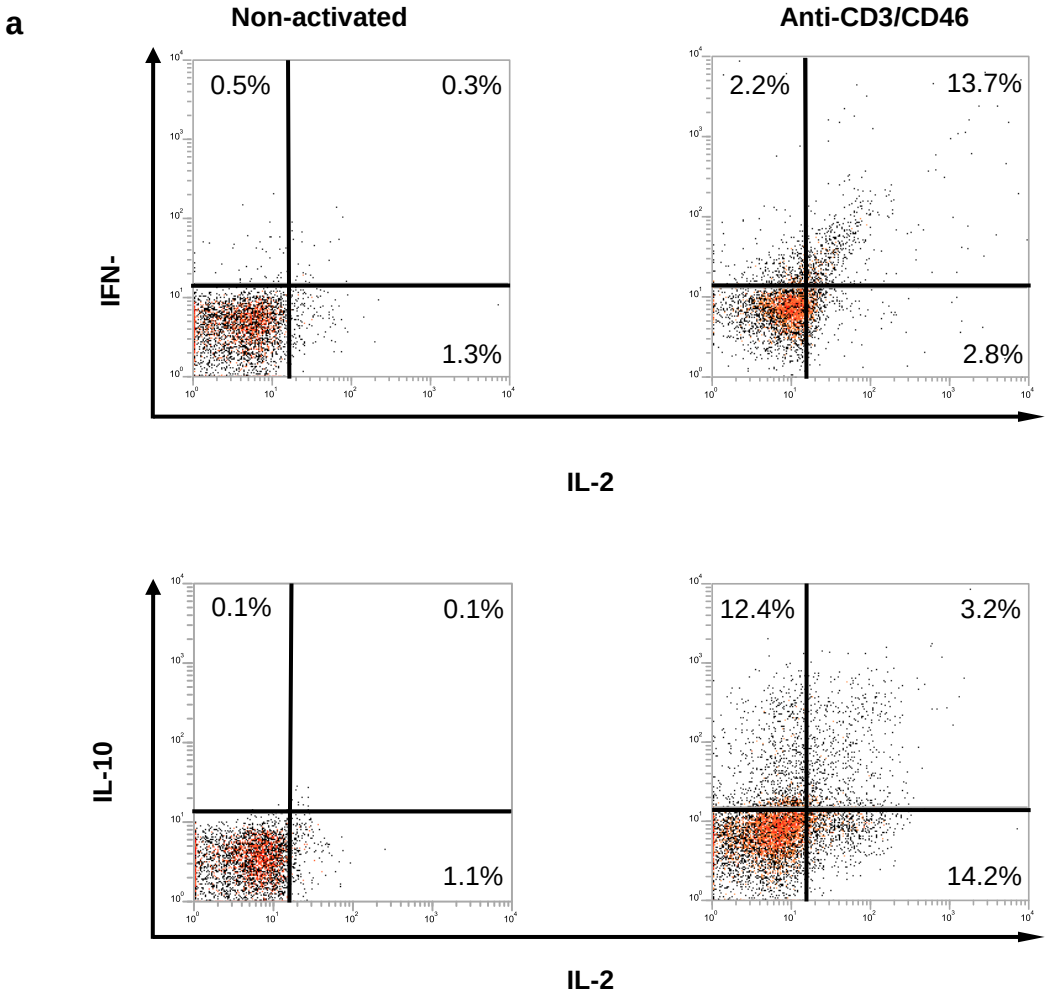


Figure 3.11 CD3/CD46-induced IL-10 production is coupled with inhibition of IL-2 secretion. Purified $CD4^+$ T cells were either left non-activated or stimulated with plate bound mAbs to CD3 and CD46 (Anti-CD3/CD46) for 36 hours and active secretion of IFN-γ, IL-2 and IL-10 was measured through secretion assays. Top dot plots refer to the secretion assay conducted for the evaluation of IFN-γ and IL-2 secretion using IFN-γ (FITC) and IL-2 (APC) as detection antibodies in non-activated and CD3/CD46-activated T cells. Lower dot plots refer to the secretion assay for the identification of IL-10 (PE) and IL-2 (APC) in non-activated and CD3/CD46-activated T cells. Values represent the percentage of positive cells within the relevant quadrant. Results are representative of three similarly performed experiments.

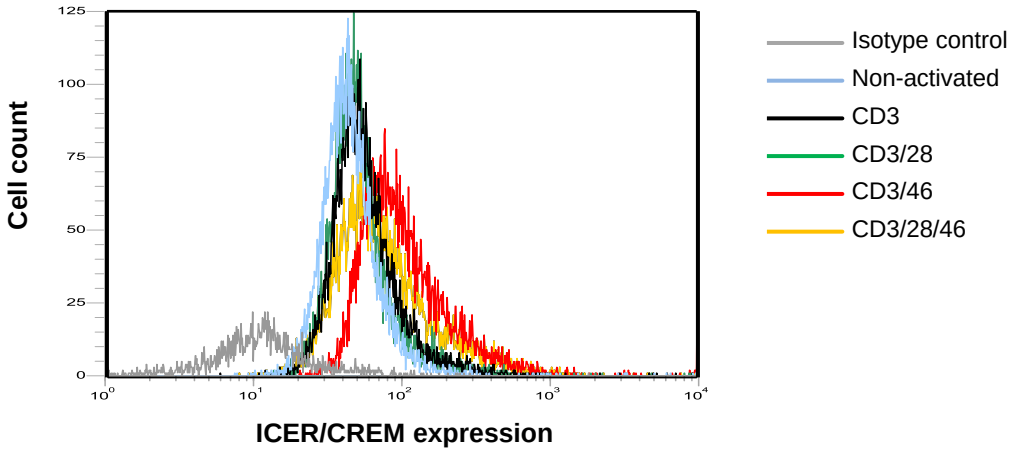


Figure 3.12 CD3/CD46-activation increases ICER/CREM expression. a) Purified CD4$^+$ T cells were either left non-activated or stimulated with plate bound antibodies to CD3, CD3 plus CD28 (CD3/28), CD3 plus CD46 (CD3/46) or CD3 plus CD28 plus CD46 (CD3/28/46) used at a oncentration of 2 μg/ml. T cells were stimulated for 48 hours in the presence of 50 U/ml of rhIL-2. Following stimulation T cells were harvested and assessed for ICER/CREM expression by intracellular staining. Data are reported as histograms of mean fluorescence intensity (MFI) of expression and are representative of 3 experiments.

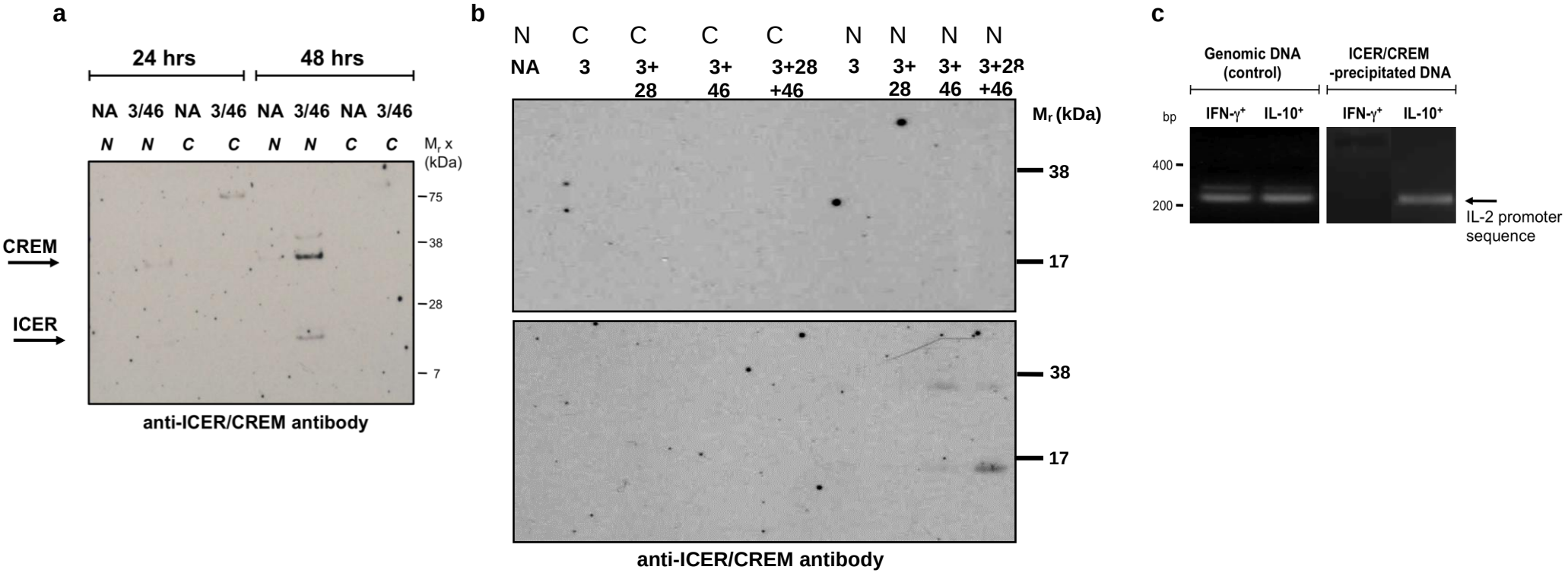


Figure 3.13 CD46-mediated signals participate in the regulation of IL-2 expression by CD4+ T cells via nuclear translocation of ICER/CREM. a) Purified CD4+ T cells were either left non-activated (NA) or were activated with plate bound mAbs to CD3 plus CD46 (CD3/46) in the presence of 50U/ml of rh-IL2 for 24 and 48 hours. Nuclear (N) and cytoplasmic (C) protein fractions were obtained at each time point. Normalized protein samples were analyzed for ICER/CREM expression by Western blotting. Shown is one representative of three experiments. b) Purified CD4+ T cells were activated as in (a). In addition CD3, CD3/28, CD3/28/46 activations were performed for 24 (top panel) and 48 hours (bottom panel). T cells were activated with mAbs to CD3 plus CD46 (2μg/ml) for 36 hours in the presence of 50 U/ml IL-2. Subsequently IFN-γ+/IL-10(-) cells and IFN-γ(-)/IL-10+ cells were then FACS sorted and 1.2 x10^5 cells for each population were used for chromatin immunoprecipitation using a mAb to ICER/CREM and IL-2 promoter specific primers. The left panel shows a PCR control reaction using the genomic DNA of both T cell populations and the right panel shows the PCR reaction using the respective DNA samples following the chromatin immunoprecipitation procedure. Data shown are representative of three independently performed experiments.

3.8 Defective IL-10-switching in rheumatoid arthritis

As mentioned previously, assessing the *in vivo* importance of CD46-mediated regulation of T_H1 responses is currently hampered by the lack of a suitable small animal model. Thus, T cells from patients that suffer from a T_H1-mediated autoimmune disease, rheumatoid arthritis (RA), were investigated to assess whether they present a defect in the CD46-mediated regulation of the T_H1 induction and contraction pathway. The responses to CD3/CD46 activation in the presence of 25 U/ml IL-2 of $CD4^+$ T cells from three healthy donors and three adult patients with inflammatory arthritis were compared. After 36 hours of activation the cultures from RA patients contained higher percentages of IFN-γ+/IL-10(-) and IFN-γ+/IL-10+ but very few and IFN-γ(-)/IL-10+ T cells (Figure 3.14). In addition, while amounts of IFN-γ and IL-10 in the supernatants of T cells from healthy donors were roughly similar, T cells from RA patients produced ≥10 fold more IFN- γ than IL-10 (Figure 3.14b). Because chronic inflammatory conditions such as RA are often attributed to inappropriate responses to persistent antigen, cytokine expression was examined after T cell re-stimulation with CD3/CD46. Cultures of T cells from healthy donors showed increased switching to the IFN-γ(-)/IL-10+ state, now containing approximately equal distributions of IFN-γ-producing (IFN-γ+/IL-10(-) and IFN-γ+/IL-10+ and IL-10-secreting (IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+) T cells (Figure 3.14c). In contrast, re-stimulated cultures from RA patients lacked a significant IFN-γ(-)/IL-10+ population, with a diminution even in IFN-γ+/IL-10+ cells relative to the cells' initial stimulation (Figure 3.14c). To further investigate IL-10-switching in arthritis, $CD4^+$ T cells were isolated from the synovial fluid of both inflamed knee joints of a juvenile arthritis patient. Similar to the results obtained with blood-derived $CD4^+$ T cells from RA patients, these synovial T cells did not switch to the IFN-γ(-)/IL-10+ state after an initial CD3/CD46 stimulation (Figure 3.14d); produced significantly more IFN-γ than IL-10 during expansion (Figure 3.14e); were mostly 'locked' into the IFN-γ+/IL-10(-) state upon restimulation (Figure 3.14f); These data strongly suggest that the TCR/CD46/IL-2-mediated switch from a pro-inflammatory T_H1 to an immunoregulatory IL-10-secreting phenotype is dysfunctional in RA and juvenile arthritis patients. A different explanation to these set of data is linked to the kinetics of IL-10 expression and secretion in RA patients and healthy donors. It may be possible that $CD4^+$ T cells from RA patients require longer activation through CD3 and CD46

signals in order to start producing IL-10 in higher amounts that could counteract the IFN-γ production. To address this point $CD4^+$ T cells from RA patients could be activated for longer than 36 hours and cytokine secretion screened at different time points in a kinetic study. A different explanation for the low frequencies of IL-10 producing cells could be linked to epigenetic changes of the IL-10 gene. The DNA methylation status (at CpG sites) is associated with cytokine expression (Fitzpatrick and Wilson, 2003). The IL-10 gene could present a hypermethylated status in $CD4^+$ T cells of RA patients which would not allow for its expression. A further possibility that could explain the lower IL-10 production by RA patients in comparison to healthy donors is the inability of $CD4^+$ T cells from RA patients to shutdown IFN-γ production; as a consequence the ratio between IFN-γ and IL-10 would strongly favour IFN-γ and this could negatively impact on the switch favouring the IFN-γ phase.

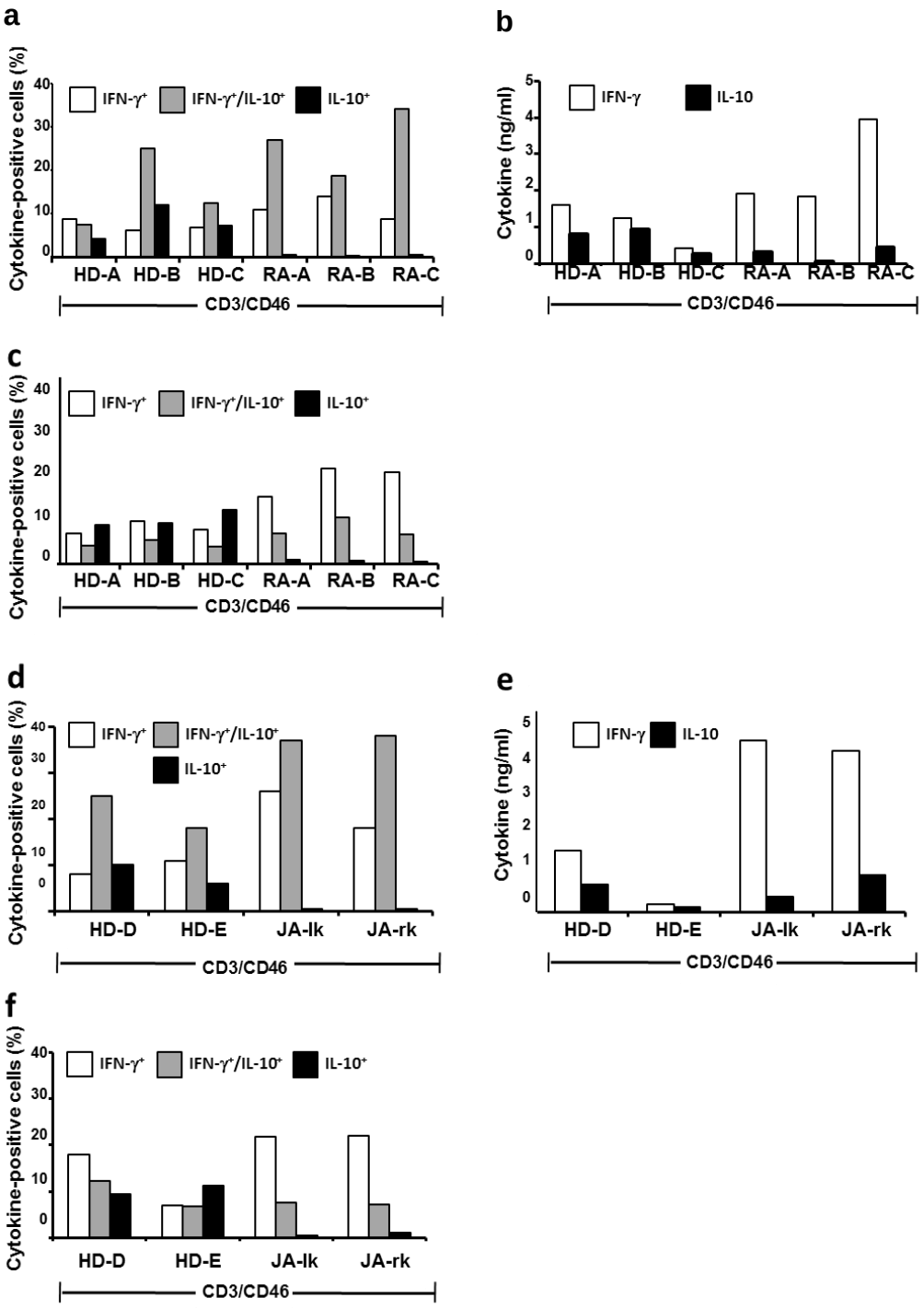


Figure 3.14 CD4$^+$ T cells from patients with rheumatoid arthritis are defective in the IFN-γ (T_H1) to IL-10 switch induced by anti-CD3 and anti-CD46. (a) Comparison of cytokine-secreting cells among blood-derived T cells obtained from three healthy donors (HD-A–HD-C) and three patients with rheumatoid arthritis (RA-A–RA-C) and activated for 36 h with anti-CD3 and anti-CD46 plus IL-2 (50 U/ml). (b) IFN-γ and IL-10 in supernatants of T cells activated as described in (a) and then maintained in culture for 5 days with IL-2 (50 U/ml). (c) Frequency of cytokine-secreting cells among cells treated as described in (b), then re-stimulated for 8 hours

with anti-CD3 and anti-CD46 plus IL-2 (50 U/ml); results presented as in (a). (d) Frequency of cytokine-secreting cells among T cells from the synovial fluid of the left knee (JA-lk) and right knee (JA-rk) of a patient with juvenile arthritis and blood-derived T cells from two healthy donors (HD-D and HD-E) after primary stimulation with anti-CD3 and anti-CD46; results presented as in (a). (e) Secretion of IFN-γ and IL-10 by the cells in d during population expansion as described in (b). (f) Frequency of cytokine-secreting cells among the cells in d after secondary stimulation as in c. Data are representative of two experiments (a–f). ^Data generated by Dr Claudia Kemper and Dr Gaelle Le Friec.

3.9 Local complement production drives IL-10 expression during T cell activation

In the experimental *in vitro* system used, activation of $CD4^+$ T cells was achieved by immobilised antibodies to CD3 and CD46. Engagement of the TCR *in vivo* is easy to envision as it is achieved through the presentation of peptide loaded MHCII by APCs. A more complicated scenario to envisage regards the engagement of CD46 during T cell activation. It could be argued that presence of a pathogen will activate the complement system and thereby lead to generation of C3b (the prime ligand for CD46) on the activated T cells. However, a more intriguing possibility is suggested by publications describing the generation of local complement activation fragments during cognate APC-T cell interactions in mouse systems (Heeger et al., 2005; Lalli et al., 2007; Strainic et al., 2008). To assess whether local complement fragments are also produced by human $CD4^+$ T cells, the hypothesis according to which TCR-activation can locally induce CD46 ligands was investigated. Activation of $CD4^+$ T cells with anti-CD3 mAbs showed high C3b deposition onto T cells surfaces (Figure 3.15a). C3b generation was increased by co-stimulation with either CD28 or CD46 mAbs attesting the ability of TCR and CD46 engagement to locally induce CD46 ligands/signalling. A second important question that arose from these studies concerned the importance of CD46 signalling in comparison to the 'classic' co-stimulator CD28 in the IL-10 induction pathway of human T_H1 cells. In order to address this question, $CD4^+$ T cells were purified via magnetic sorting. Subsequently Dr. Kemper activated the purified T cells through CD3- and CD3/CD28-activated in the presence of soluble recombinant CD46; this allowed locally produced C3b, generated upon TCR activation, not to bind cell membrane expressed CD46 and prevent signalling. Surprisingly, blocking endogenous CD46 engagement (by saturating the system with soluble CD46) determined a decrease of IL-10 production in both anti-CD3 and anti-CD3/CD28 activated T cells. These data demonstrated that the activation of T cells provides the means to engage CD46 locally. In addition it confirmed that CD46, and not CD28, is the key co-stimulator for the IL-10 switch.

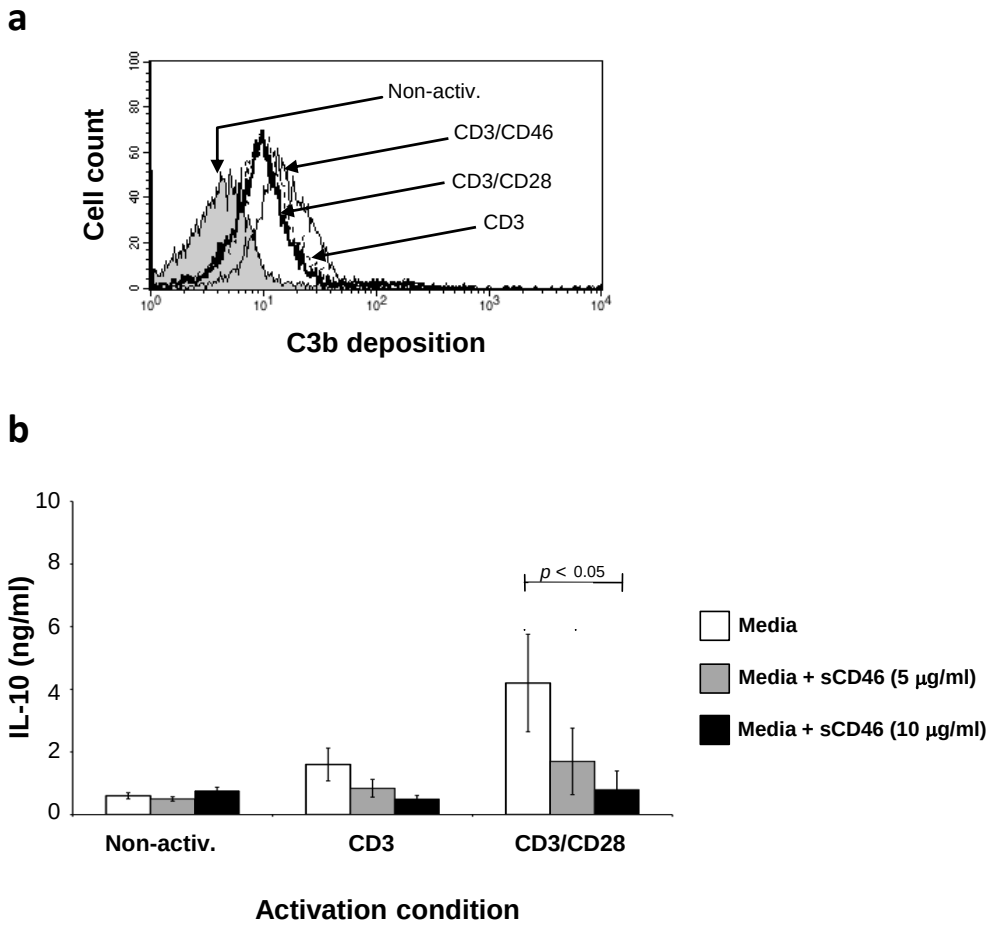


Figure 3.15 CD46 induced C3b drives IL-10 expression in CD4+ T cells stimulated with anti-CD3 and anti-CD28. (a) Flow cytometry of C3b surface deposition on purified CD4+ T cells either non-activated or activated for 12 hours with immobilized mAbs to CD3, CD3 plus CD28 and CD3 plus CD46 (2 μg/ml). Data are representative of four experiments. (b) Production of IL-10 by purified T cells either left non activated or activated for 36 hours with mAbs to CD3 alone or mAbs to CD3 plus CD28 (horizontal axis) plus media alone or media plus soluble CD46 (sCD46). *p < 0.05 derived from Friedman's test with Dunn's multiple comparisons. ^ Data generated by Dr Claudia Kemper.

3.10 Discussion

The increasing evidence for the importance of IL-10 in suppressing immunopathology is clearly documented in humans, where it has both biological and clinical implications (He et al., 2009). Despite the identification of IL-10 secreting Tr1 cells more than a decade ago, the inability to identify a marker/transcription factor associated to such cells has hampered progress in this field of research (Bacchetta et al., 1994; Groux et al., 1997). By contrast increasing evidence supports the concept of a switching model through which IL-10 secreting cells derive from an earlier incarnation of IFN-γ producing T_H1 cells. Cultures that co-express IL-10 and IFN-γ have been clearly documented (Arase et al., 1994; Meisel et al., 1996; Taub et al., 1993a), and such a switch would have the advantage of inducing a regulatory cytokine and simultaneously suppressing an effector cytokine based on the recognition of the same antigen. The data shown in this chapter prove that human T_H1 cells are promoted by the co-engagement of the TCR and CD46. Increasing environmental IL-2, as could be envisaged during an effector response (Figure 3.16), promotes the switch of T_H1 cells to IL-10 producing lymphocytes and concomitantly diminishes endogenous IL-2 production via nuclear translocation of ICER/CREM.

The temporally regulated induction of cytokines with opposing effects (i.e. IFN-γ and IL-10) mediated by CD46 clarifies the controversial findings regarding the ability of CD46-induced T cells to support DC maturation and B cell responses (Barchet et al., 2006). In fact, although it is known that Tr1-derived IL-10 negatively affects DCs maturation by preventing the up-regulation of MHCII, CD80 and CD86, supernatants from CD46-activated T cells have been shown to be able to promote DC maturation, antigen presentation and T cell induction (Barchet et al., 2006). The latter effects were described in the original report by Barchet et al., (2006) as mediated by the cosecretion of granulocyte–macrophage colony-stimulating factor (GMCSF) and soluble CD40 ligand (sCD40L) which neutralize the CD46-indued IL-10 inhibitory effects on DCs (Steinbrink et al., 2002). However, in light of the successive induction of the CD46-mediated T_H1 and IL-10 phases described in this chapter it is possible to speculate that mainly the T_H1 phase of the switch (i.e. IFN-γ+/IL10-) would favor DC growth and maturation through the cosecretion of GMCSF and sCD40L. The accumulation of IL-2 would then promote CD46-mediated IL-10 production during the double positive IFN-

γ+/IL10+ and single positive IFN-γ-/IL10+ phases and would inhibit DCs function favoring the contraction of the immune response. Co-cultures of CD46-activated T cells have been shown to provide B cell help enhancing antibody production (namely IgG1 and IgM) in a cell-cell contact dependent mechanism (Barchet et al., 2006). The importance of this mechanism has been shown *ex vivo*; in fact T cells from a CD46 deficient patient were not able to promote B cell help. The finding that CD46 induced signals primarily induce a T_H1 response are in accordance with the data of Fuchs et al., (2009) and support the notion that T_H1 are pivotal helpers of B cells responses and that defects affecting T_H1 induction impair humoral immunity (Fremeaux-Bacchi et al. 2006).

It is now widely acknowledged that the complement system functions well beyond a simple danger recognition and microbial clearance system and participates actively in shaping adaptive immune responses (Carroll, 2004). In recent years the ability of complement to instruct the adaptive immune response has reinforced a novel concept: the one of local complement production. Thanks to the ability of bone marrow derived cells to produce complement components, studies utilising bone marrow chimeras have been essential in elucidating the role of local complement production and in discerning it from the role of systemic complement (Strainic et al., 2008; Pavlov et al., 2008; Zhou et al 2011). Carroll's group was among the first to show the importance of C3 derived from myeloid cells of WT bone marrows in restoring humoral responses within a $C3^{-/-}$ animal (Verschoor et al., 2001). Strainic et al., described how local complement production is able to generate alternative pathways components (namely C3, fB, fD, C5) that can actively shape T cell immunity *via* APC (Strainic et al., 2008). The data shown in Figure 3.15 suggests that the engagement of human $CD4^+$ T cells through the TCR alone or in conjunction with CD46, promotes the production of C3b and its binding to CD46 showing for the first time that local complement production may be an important process also in human systems. This finding also raises a vital question regarding the hierarchy of CD46 activation in relation to CD28 costimulation in the induction of IL-10 by T_H1 cells. It is important to note that the concomitant activation of CD28 and CD46 on human $CD4^+$ T recapitulated (if not with a stronger phenotype) the cytokine secretion pattern of TCR and CD46 activated T cells (Figure 3.5). Hence, hypothetically engaging CD28 (through APCs B7-1) and CD46 (through local complement) would still favour the CD46-induced T cell switching model.

Importantly CD3/CD28 activation of $CD4^+$ T cells induces C3b deposition and the complete inhibition of CD3/CD28-induced IL-10 production by interference with 'intrinsic' CD46 activation (Figure 3.15) suggests a dominant role for CD46 in IL-10 expression by $CD4^+/T_H1$ cells. Such 'physiological' CD46 engagement through C3b/C4b produced by activated T cells may provide the molecular mechanisms by which IL-10-secreting Tr1 cells are induced *in vitro* by other groups. In fact, the generation of 'classic' adaptive IL-10 (and IFN-γ)-producing Tr1 cells in culture requires minimally repetitive CD3/CD28 activation (or exposure to DCs) of $CD4^+$ T cells in the presence of high IL-2 (Groux et al., 1997; Jonuleit et al., 2000), which are conditions conducive to CD46 engagement and CD46-induced IL-10 production.

Testing the notion that CD46 is important in regulating $CD4^+$ T cell responses in a small animal model is currently hampered by the fact that rodents lack CD46 expression on somatic cells and that a murine molecule recapitulating CD46's role in TCR/IL-2-dependent IL-10 induction has not yet been identified (Kemper and Atkinson, 2007). A previous study in humans, however, connects significantly decreased IL-10 expression upon CD46 activation of $CD4^+$ T cells with multiple sclerosis (MS) (Astier et al., 2006). Although the switch from the T_H1 to Tr1 phase was not examined in this study, low IL-10 production by T cells from MS patients were shown upon CD46 engagement. These data combine with the novel observation presented in Figure 3.14 that T cells from RA patients are unable to promote the CD46-mediated T_H1/Tr1 switch implicating defects in this pathway in a clinically significant autoimmune/inflammatory condition. The importance of T_H1 switching *in vivo* is suggested by the observation that only continuous exposure of non-allergic beekeepers to high doses of bee venom induces a decrease of antigen specific IFN-γ-secreting T_H1 cells and a parallel increase in antigen specific IL-10-producing Tr1-like cells indicated by the decrease of T cell-mediated cutaneous swelling (Meiler et al., 2008). After antigen withdrawal, bee venom-specific T cells produce again only high IFN-γ and no IL-10 upon first re-activation and increased IL-10 production by T cells only occurs upon repetitive stimulation. Although the data do not prove that the same antigen specific T cells producing IFN-γ are switching to IL-10 production, the authors suggest that this switch mechanism may be a viable one. The data presented in Figure 3.7 showed that naive CD46-activated T cells follow a similar scheme by which they acquire an evident Tr1-like phenotype only upon repetitive stimulation. These data are

consistent with the recent concept that IL-10-secreting cells may not necessarily represent a lineage but rather the 'endpoint' of a successful effector T cell response.

The model in Figure 3.16 summarizes and envisages how CD46 signals may be involved in the shaping of an immune response against pathogens inducing a T_H1 response (i.e. TB, Leishmania, Toxoplasma). During the "initiation phase" of an immune response set to eliminate a pathogen, TCR engagement through the pMHC II of APCs, would favour C3b production and consequently CD46 engagement; given the low micro-environmental IL-2 characteristic of the initial phase of an immune response (due to low T cell numbers), the $CD4^+$ T cells would skew towards a T_H1 phenotype through the activation of STAT4, STAT1 and T-bet. During the "effector phase" of the immune response, intermediate/high environmental IL-2 derived from the expansion of T cells, would be implemented by CD46-activated T cells as the signal for the induction of IL-10 secretion and IFN-γ shutdown. IL-10 Tr1 cells (Foxp3-) would then drive the contraction after the pathogen has been cleared by inhibiting bystander T_H proliferation, IL-2 and IFN- γ production and by negatively regulating DCs. Different approaches could be taken to verify the model in figure 3.16. Silencing CD46 in human T cells would represent an approach to prove the importance of the signalling through this molecule for the initiation of a T_H1 response and for the production of IL-10. Co-cultures of DCs and T cells in which CD46 is not expressed (by RNA silencing or by using T cells from CD46 deficient patients) could be compared to co-cultures of DCs and CD46-expressing T cells. Evidence showing the involvement of CD46 in T_H1 induction and switching could be provided by screening the amounts of secreted IFN-γ and IL-10 together with the expression of T_H1 markers T-bet, STAT1 and STAT4 in the two different co-culture systems.

Given the central role of IL-2 in the switch model proposed in figure 3.16, the importance of CD46 and IL-2 signals could be evaluated using co-cultures of DCs and T cells deficient for IL-2. In such experiments, $CD4^+$ T cells would be activated through CD46 with mAbs and subsequently co-cultured with DCs. C3b could be used as a stimulus to activate $CD4^+$ T cells through CD46 but the use of C3b as stimulus could have confounding effects since it would bind to CR1. CR1 engagement on T cells, negatively regulates proliferation and activation (Wagner et al., 2006). Co-culturing CD46 activated T cells and DCs in an IL-2 deficient system would provide a setting of complete dependency on exogenous IL-2. Hence, CD46-activated T cells would be activated through TCR engagement by DCs and adding exogenous IL-2

could be an approach to test the model in figure 3.16. In fact, adding low, intermediate or high concentrations of IL-2 would enable to determine the generation of mainly IFN-γ producing T cells (low IL-2) or IFN-γ and IL-10 producing T cells (intermediate and high IL-2). Hence, the supernatants for each of these cultures (i.e. low, intermediate and high IL-2) could provide information on the production of IFN-γ, IL-10 and C3b and allow to gather information on whether these components would be produced in a similar trend to the one shown in figure 3.16. Since one of the main issues in proving the model in figure 3.16 is the demonstration that the same T cell that responds to a specific antigen switches from the production of IFN-γ to the one of IL-10, a technically challenging approach, would involve the generation of T cells with the genes for IFN-γ and IL-10 under different fluorescent proteins (e.g. in such system IFN-γ production could be detected through the expression of green fluorescent protein). An imaging experiment involving DCs and CD46-activated T cells co-cultures could provide evidence of the different cytokines produced in different time points of a responding T cell. Importantly, the absence of a small animal model poses great limitations for the study of the model in figure 3.16 in an *in vivo* setting of infection.

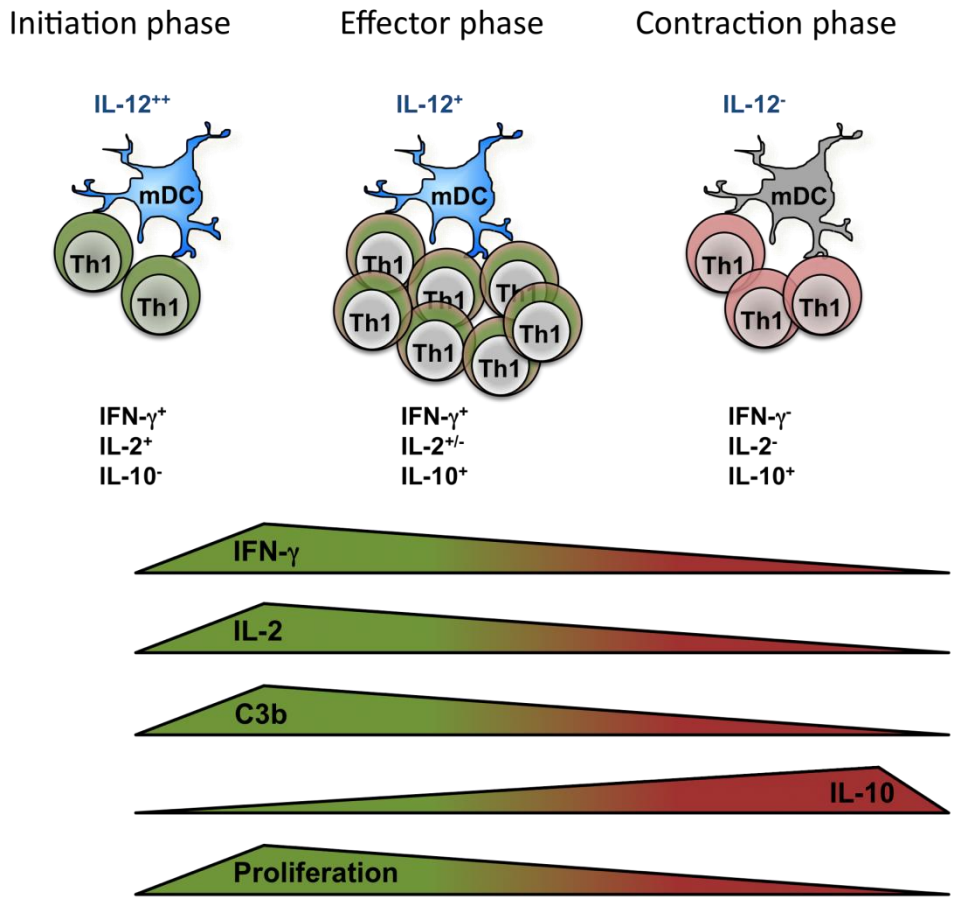


Figure 3.16 CD46 and IL-2 mediated signals in the self-regulating loop of T_H1 lymphocytes. Representative figure of the implementation of T cell incoming signals induced by myeloid Dendritic Cells (mDCs) (TCR, signal 1), varying concentrations of micro-environmental IL-2 (Signal 2) and C3b (Signal 3) developed during an immune response towards an intracellular T_H1-inducing pathogen (Kemper et al., 2011).

Chapter 4

Investigating the molecular signatures of the CD46-mediated T_H1 regulation

4.1 Introduction

The mechanism of T cell switching described in Chapter 3 ensures the important flexibility to respond appropriately to micro-environmental signals during an infection setting. However, it poses a major obstacle in the therapeutic usage of IL-10-secreting T_H1 suppressor cells; in fact suppressive IL-10 T cells generated in a controlled *in vitro* environment may reacquire a pro-inflammatory T_H1 phenotype after injection into autoimmune or transplant patients. Thus, a better understanding of the intracellular pathways driving the IFN-γ to IL-10 switch is necessary to provide potential targets to either induce or abrogate specific cytokine secretion in T_H1 cells. During the next part of the thesis, the study concentrates on the definition of the molecular signature accompanying cytokine switching in human T_H1 cells. In order to clearly define the molecular signals regulating the CD46/IL-2-mediated 'life cycle' of T_H1 cells and highlight key molecules involved in cytokine switching, gene arrays from the three distinct IFN-γ and/or IL-10 expressing T cells were generated (see paragraph 4.3 and figure 3.16). The asparagine endopeptidase (AEP) was identified as among the highest differentially expressed genes between these populations. AEP is a lysosomal cysteine endopeptidase with a wide range of immunological functions including antigen processing, MHC II and TLR maturation (Sepulveda et al., 2009; Watts et al., 2003). The human homologue of AEP, firstly identified in legume seeds (Chen et al., 1997), was cloned and discovered in 1997 by Chen. The gene that encodes for human AEP is located on chromosome 14q32.1 (NCBI, Nucleotide) and encodes for a protein of 433 amino acids (Chen et al., 1997). AEP is translated as an inactive precursor which undergoes proteolytic cleavage in order to generate the active form of the peptidase (Li et al., 2003). AEP maturation requires two steps; the inactive form of 56 kDa located in pre-lysosomal compartments, is auto-catalytically converted into an active precursor of 46 kDa after the sequential cleavage of a C-terminal 110 residue and an N-terminal 8 residue propeptide (Li et al., 2003). Auto-activation is a pH dependent process and is optimal for pH values ranging from 4.5 and 5.5 which are achieved during the endo/lysosomal maturation process. The second step is not autocatalytic but mediated by different lysosomal proteases and generates a mature 36 kDa form (Li et al., 2003). Proteases are classified on the basis of the key amino acid found in the active site. They include Serine, Cysteine, Threonine, Aspartate and Glutamic proteases (Rawlings et al., 2012). AEP is a unique cysteine protease as it

specifically cleaves proteins after an asparagine (Asn) residue. AEP is a member of the peptidase family C13. Most of the known cysteine proteases, including cathepsins S, L, F, B and C are related to the enzyme papain and are grouped in the C1 family of peptidases (Chen et al., 1998; Rawlings et al., 2012; Watts et al., 2005).

Initial data regarding the immunological role of AEP focused mainly on the antigen processing pathways. Manoury et al. (1998), showed that inhibition of AEP *in vitro* and *in vivo* affects the processing of tetanus toxin antigen and its presentation in association with MHC class II molecules in B cells (Manoury et al., 1998). Further studies highlighted how the enzymatic activity of AEP induces the destruction of auto-antigens such as MBP, playing an important role in protection from autoimmunity (Manoury et al., 2002). The finding that AEP is involved in the removal of the MHC Class II Invariant Chain Chaperone during the MCH class II maturation process reinforced the importance of this molecule in APCs. However, further *in vivo* analyses showed that AEP is not essential in the class II antigen presentation process since it shares redundancy of function with other lysosomal proteases (Maher et al., 2005). AEP mediates the initial processing of TLR3, TLR7 and TLR9 in conjunction with members of the cathepsins families (Sepulveda et al., 2009). Studies on AEP knockout mice showed that AEP is also important in the maturation of the lysosomal cystein protease known as Cathepsin (Cat) L. Maeher et al. (2005) showed that AEP-deficient cells fail to develop the mature two chain form of Cat L which is important in the development of $CD4^+$ T cells. However, the authors reported that AEP knockout mice have normal numbers of $CD4^+$ T cells when compared to WT mice (Maehr et al., 2005). A second study on AEP knockout mice showed the development of a group of symptoms that resemble the human disease Hemophagocytic Lymphohystiocytosis which is characterised by an increase of lymphocytes and hystiocytes (i.e. macrophages and DCs). Pathological symptoms of spleno- and hepato-megaly were reported together with an increased level of TNF in the blood. Other cytokines including IFN-γ, IL-1β and IL-4 were not affected by the absence of AEP (Chan et al., 2009). Together these data highlight how AEP has multiple immune related functions that can affect innate (e.g. TLR) sensing and adaptive initiation of immune responses (i.e. *via* APCs).

4.2 Hypothesis and Aims

Most of the data available on AEP is related to its function in antigen processing with particular focus on APCs (B cells, DCs and macrophages). Although Maher's paper showed that $AEP^{-/-}$ mice have normal numbers of $CD4^+$ T cells, the study did not contain any subsequent functional assays in regards to cytokine production by activated mouse T_H1 cells. In fact, the field currently dismisses a direct role for AEP in T cell function. Given the existence of a link between CD46 signalling and AEP expression, shown by gene array data on CD46-induced IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells, it was interesting to test the hypothesis by which AEP is a molecular regulator of IFN-γ and IL-10 production or secretion by human-CD46 activated T cells. Therefore, the main aims were to confirm AEP expression on a gene and protein level, analyse the effects of AEP inhibition on human T cell activation and assess the impact of AEP in the switch of CD46-activated human T cells.

4.3 Generation of gene arrays from CD46 induced IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells

To understand the molecular signals regulating the CD46/IL-2 mediated switch of human T_H1 into IL-10 regulatory cells, gene arrays were generated using mRNA isolated from the three distinct populations of IFN-γ and/or IL-10 secreting T cells (IFN-γ+, the IFN-γ+/IL-10+ and IL-10+). To obtain representative results and statistical significance two donors were selected for each gender and were aged matched (Table 4.1). Given that naïve and memory T cells respond similarly to CD3/CD46/IL-2 activation in regards to IFN-γ/IL-10 switching (Figure 3.7), and in order to obtain at minimum 10^6 cells post sort for each of the three populations, whole $CD4^+$ T cells were blood purified and activated through antibodies to CD3 and CD46 in the presence of rhIL-2 (50 U/ml) for 36 hours. Following activation a secretion assay was performed in order to identify and sub-sort the IFN-γ producing cells, the IFN-γ/IL-10 co-secreting cells and the IL-10 secreting lymphocytes (Figure 4.1). Post sort the mRNA was isolated and screened for purity using a Nanodrop ND-1000 spectrophotometer (Table 4.2).

	Age	Sex
Donor 1	48	Male
Donor 2	37	Male
Donor 3	32	Female
Donor 4	45	Female

Table 4.1 Gene array donors, age and sex. The table reports the age and sex of the donors involved in the gene arrays study. From each donor $CD4^+$ T cells were magnetically sorted via positive selection and subsequently activated with anti-CD3 plus anti-CD46 mAbs in the presence of 50 U/ml of rhIL-2 for 36 hours.

a

b

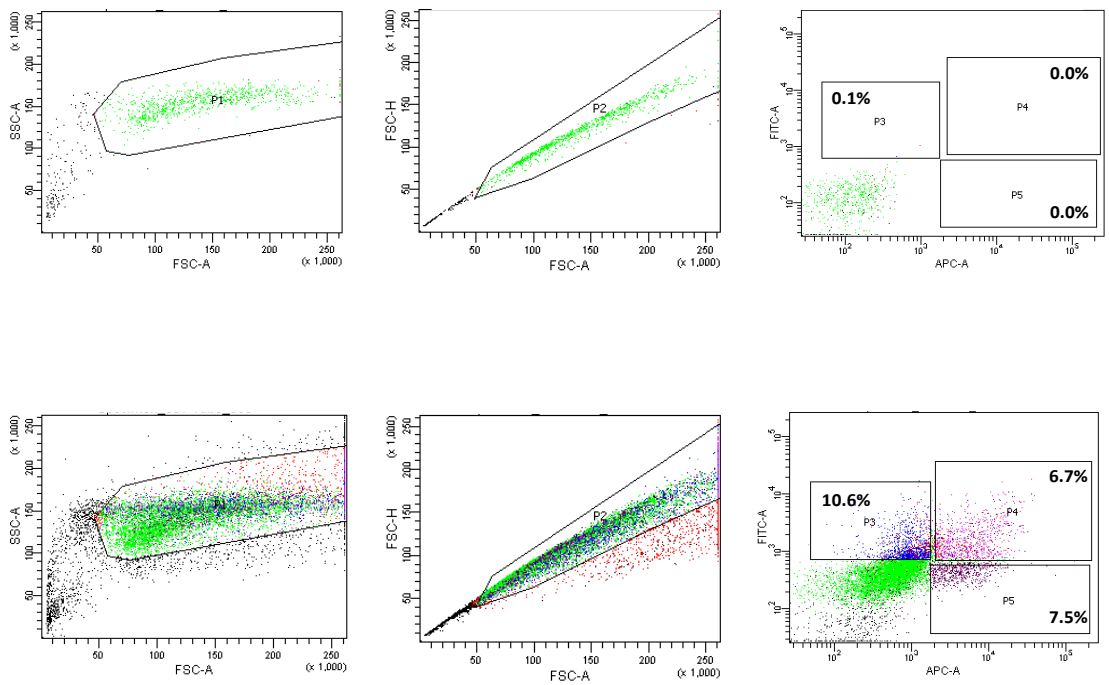


Figure 4.1 Sorting strategy for the isolation of IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells induced by anti-CD3 plus anti-CD46 activated T cells. $CD4^+$ T cells were isolated from 4 different donors (Table 4.1) and activated with mAbs to CD3 and CD46 (2 μg/ml) for 36 hours in the presence of 50 U/ml of rhIL-2. Following activation IFN-γ and IL-10 secretion assays were performed for each donor, in order to identify and sub-sort the three populations of IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ secreting cells. The gating strategy adopted for the sorting of IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ lymphocytes was based on FSC/SSC gating (gate P1, left panel), doublet depletion (central panel, gate P2) and IFN-γ (FITC-A) and/or IL-10 (APC-A) positive staining (right panel, gates P3-blue-, P4 –pink- and P5 –violet-). Unstained (a) and stained (b) samples are shown. Numbers indicate percentages of positive cells within the relevant gate.

RNA Sample	RNA ng/µl	Absorbance Ratio 260/280
IFN-γ+ (Donor 1)	282	2.08
IFN-γ+/IL-10+ (Donor 1)	128	2.09
IL-10+ (Donor 1)	135	2.06
IFN-γ+ (Donor 2)	66	2.03
IFN-γ+/IL-10+ (Donor 2)	58	2.06
IL-10+ (Donor 2)	179	2.05
IFN-γ+ (Donor 3)	438	2.06
IFN-γ+/IL-10+ (Donor 3)	280	2.01
IL-10+ (Donor 3)	239	2.08
IFN-γ+ (Donor 4)	244	2.01
IFN-γ+/IL-10+ (Donor 4)	247	2.03
IL-10+ (Donor 4)	248	2.04

Table 4.2 mRNA purity screen. The mRNA extracted from IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ sorted cells of each donor was screened for purity using a Nanodrop spectrophotometer. The quality control was performed by analysis of the absorbance ratio between readings at 260 nanometers and 280 nanometers (A260/280). Ratios of A260/280 equal or greater than 2 indicated the absence of contaminants (e.g. proteins or phenols) and was indicative of high purity RNA.

The gene chip was carried out on the Affymetrix GCS3000 platform provided by the KCL genomic centre in collaboration with Dr Matthew Arno. The quality controls that were performed for the validation of the gene array data are shown in figure 4.2 and included: positive vs negative area under the curve (AUC), all probe set relative log expression (RLE) mean, all probe set mad residual mean and box plots of raw and normalised intensity signals (Figure 4.2). The positive vs negative AUC value for a receiver operating characteristic (ROC) curve, plots the detection of positive controls (putative exon based probe sets of housekeeping genes) against negative controls (putative intron based probe sets of housekeeping genes). The positive vs negative AUC plot evaluates how the probe set signals differentiate the positive from the negative controls and is a measure of overall data quality. A value of 1.0 indicates perfect separation whilst a value of 0.5 indicates no separation. Values between 0.8 and 0.9 reflect typical values (Affymetrix quality control guidelines). On the gene array performed the values were greater than 0.8 indicating coherent values for a positive quality control (Figure 4.2a). The all probe set RLE mean represents the mean absolute relative log expression and compares the signal of each probe set to the median signal value of the probe set in the study. This metric allows identification of outlier arrays. Values of samples that fall within a range of +/- 0.13 are considered non outliers (Affymetrix quality control guidelines). On the gene arrays performed in this study the all probe set RLE values were found within this range indicating the absence of outliers in the study (Figure 4.2a). The all probe set mad residual mean is the mean of the absolute deviation of the residual signal (i.e. difference between actual value and predicted value) from the median. This metric allows to identify gene chip arrays that are behaving differently from the predicted model and are highlighted by evidently greater values that do not fit within the range values of the other arrays (Affymetrix quality control guidelines). In this study the values of the all probe sets ranged between 0.23 and 0.34 and no evident outlier was identified. Further assessment of the gene

arrays quality control included the analysis of boxplots relative to signal log intensities which enable to gain information on outliers of signal intensities (Affymetrix quality control guidelines). Boxplots report $\log_2$ transformed values of non-normalised raw probe intensity (Figure 4.2b). Such analysis enables to verify the distribution of probes intensity levels between arrays. In each box, the middle line represents the median, the upper end represents the upper quartile and the lower end represents the lower quartile. Outliers are defined as values that are located more than 1.5 times the interquartile range from the edges of the box. In the gene array study carried out none of the arrays showed median values with an evident difference from the rest of the arrays indicating the absence of issues that required further validation analysis of the study. The normalisation of the probe set $\log_2$ intensities (Figure 4.2c) enabled to gain further information on outliers of signal intensities (Affymetrix quality control guidelines). The normalisation of the gene arrays in the study was carried out using the Robust Multi-Array Analysis (RMA) algorithm and upon normalisation the $\log_2$ intensity values were found to be within a smaller range than the non-normalised median values as expected in a positive outcome of quality control and excluding issues related to the normalisation process of the data.

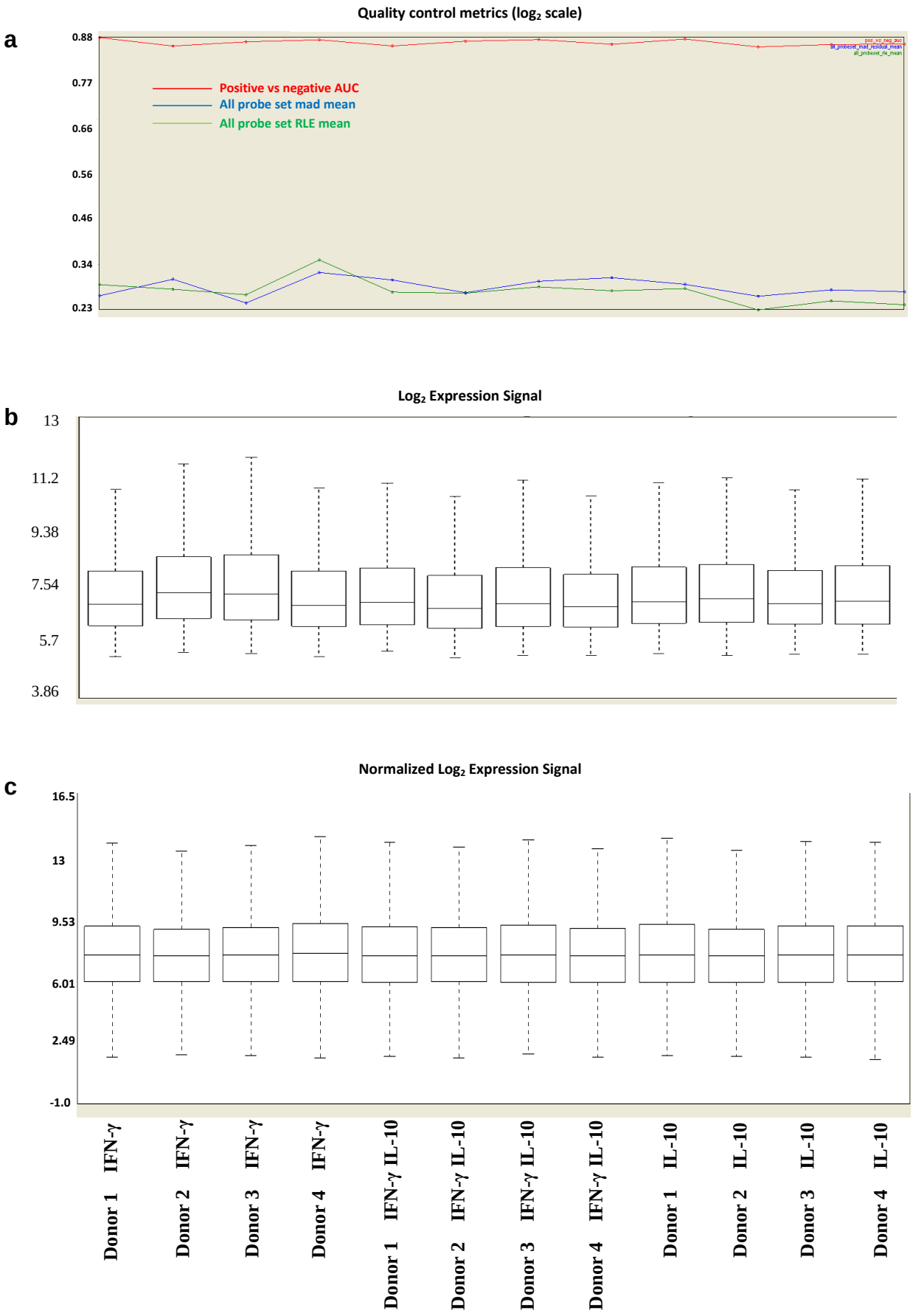
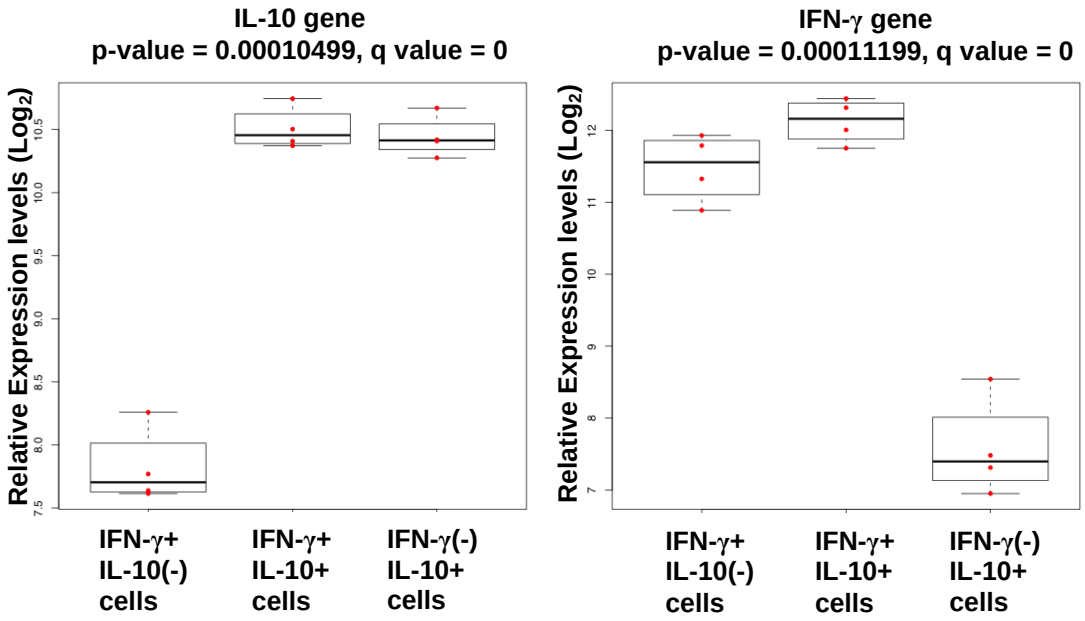


Figure 4.2 Quality control of gene arrays performed on the Affymetrix GCS3000 platform. The metrics of quality control screens for the gene array were performed for

each of the 3 cell-sorted population of IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells relative to each donor (4 donors) in order to verify the validity of the data for each of the 12 samples. a) The quality control study included analysis of the positive vs negative area under the curve (AUC) (a, red line), the all probe sets mad residual mean (a, blue line) and the all probe set relative log expression (RLE) mean (green line, a) in order to assess overall data quality, gene arrays quality and to identify possible array outliers to consider during the data analysis. Values are expressed in a $\log_2$ scale (a, b and c). Boxplots of non-normalised $\log_2$ intensity values (b) and normalised $\log_2$ intensities (c) were also drawn for each array to control for outliers of signal intensities. In each box, the middle line represents the median, the upper end represents the upper quartile and the lower end represents the lower quartile.

For the data analysis Dr Irene Rebollo Mesa utilised the Significance Analysis of Microarrays (SAM) software (Tusher et al., 2001). SAM's algorithm highlights genes with statistically significant changes in expression by performing a gene-specific t test known as "d_i" for each gene "i". A score (d) is assigned to each gene based on its change in gene expression relative to the standard deviation of repeated measurements for that specific gene. As a consequence scores that give values of fold change greater than the set threshold infer genes that are potentially significant. In this study, the analysis based on SAM computed a statistic d_i for each gene *i*, as a measure of the differences between the different population analysed of IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cells which were compared in pairs. Repeated permutations of the data were used to determine if the observed differences are larger than expected by chance in the available data. The comparison between all possible combinations of the three populations was performed. The cutoff significance was chosen to minimize the false positive rate and was measured by the false discovery rate (FDR) which was set at ≤ 0.1 (i.e. $\leq 10\%$). This was combined with a threshold change of 2-fold as a minimum fold change in gene expression. The comparisons of IFN-γ+/IL-10- versus IFN-γ+/IL-10+ cells showed that the IL-10 gene was found to be among the most down regulated genes in the IFN-γ+/IL-10(-) cells when IFN-γ+/IL-10+ were set as reference of IL-10 gene expression (Figure 4.3). Similar results were obtained when comparing IL-10 levels between IFN-γ+/IL-10(-) cells and IFN-γ(-)/IL-10+ setting IFN-γ(-)/IL-10+ cells as reference of IL-10 expression. When the IFN-γ gene expression levels were compared between IFN-γ(-)/IL-10+ cells and IFN-γ+/IL-10- cells, setting the IFN-γ+/IL-10(-) cells as reference for the expression of IFN-γ, this gene was found to be among the most down-regulated genes in the IFN-(γ-)/IL-10+ T cells (Figure 4.3). Similar results were obtained comparing IFN-γ expression between IFN-γ+/IL-10+ cells and IFN-γ(-)/IL-10+ cells with IFN-γ+/IL-10+ cells set as reference (Figure 4.3). The statistical data regarding the expression of the key genes in the switch (i.e. IFN-γ and IL-10) was indicative of the accuracy of the gene array and its validity in the identification of candidate genes and molecules with a role in the CD46 mediated molecular switch (Figure 4.3b). The heat map generated from the gene arrays further showed the expression of IFN-γ and IL-10 being higher respectively in the IFN-γ+/IL-10(-) and IFN-γ(-)/IL-10+ cell sorted populations (Figure 4.4).

a



b

Gene ID	Gene Name	Score(d)	Fold Change	q-value(%)	permut. p-value
LGMN	Legumain = Asparagine Endopeptidase	-6.755	0.234	0.000	1.050E-04
IL10	Interleukin-10	-6.056	0.162	0.000	1.120E-04
x	x	-5.227	0.263	0.000	1.120E-04
y	y	-4.559	0.380	0.000	1.120E-04
z	z	-4.404	0.328	0.000	1.120E-04

Figure 4.3 Statistical evaluation of gene array data and identification of IFN-γ and IL-10 differences in expression gene levels. a) Box plot indicating the relative expression levels of IL-10 (left box plot) and IFN-γ (right box plot) in a $\log_2$ intensity scale based on the pooled data analysed for the 4 donors. The middle line in each box represents the median, the upper and lower edges represent the respective quartiles. The q-value is the lowest FDR at which the gene is called significant. Median and quartile values of expression are relative to the comparisons between IFN-γ+/IL-10(-) versus IFN-γ+/IL-10+ or versus IFN-γ(-)/IL-10+ cells for the IL-10 gene (left box plot) and the IFN-γ+ gene (right box plot). References for each comparison are listed in paragraph 4.3 b) Down-regulated genes in IFN-γ+ compared to IL-10+ cells. The expression levels of IL-10 and asparagine endopeptidase (AEP, LGMN) were shown to be down-regulated in IFN-γ+ cells when IFN-γ+ cells and IL-10+ cells were compared and the IL-10+ population was set as reference for the comparison between the two populations. Changes in expression are expressed as a relative measure of the difference in fold change with a minimum cutoff of 0.5 (1/minimum fold change = 2). Statistical analysis derived from SAM.

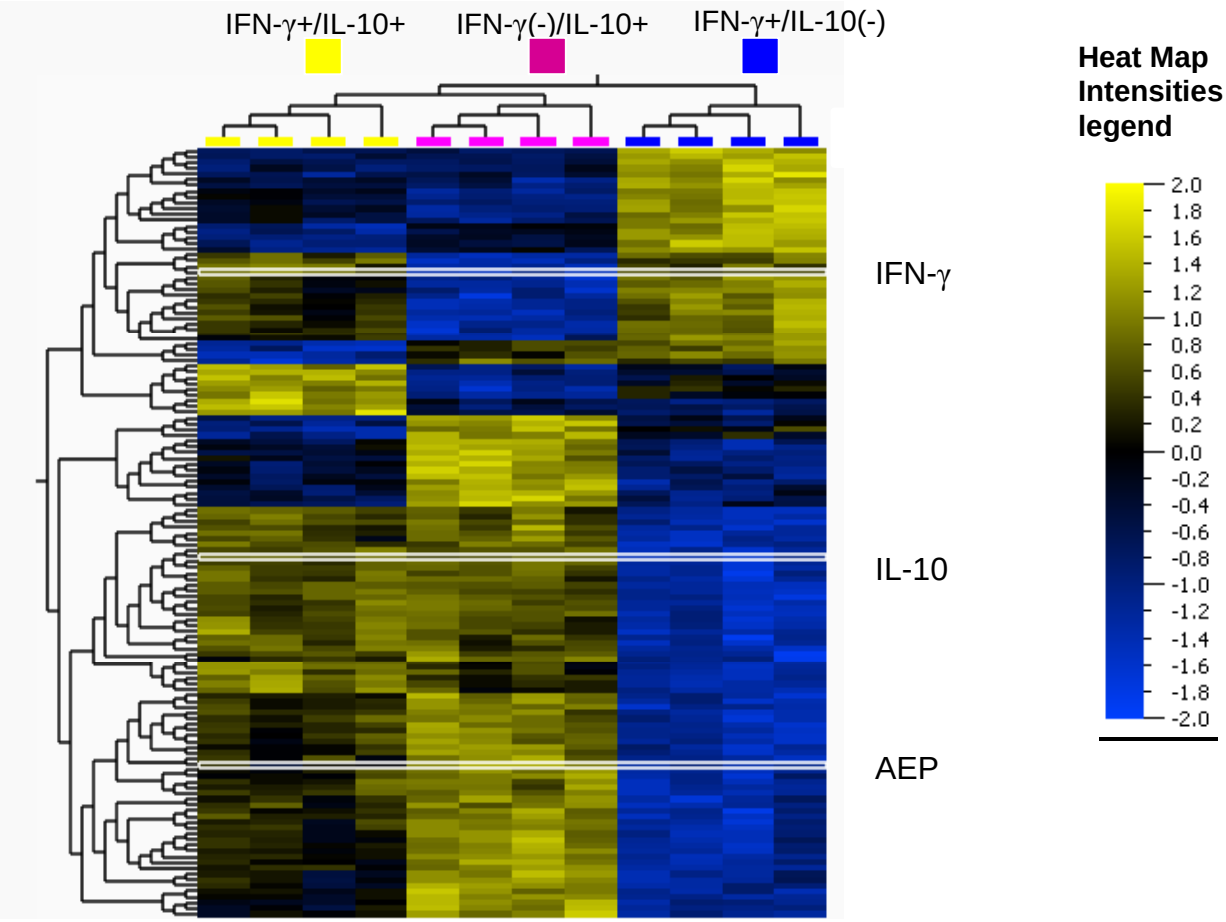


Figure 4.4 Heat map of differentially expressed genes in the IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ cell populations. The heat map generated from gene arrays run on the Affymetrix GCS3000 platform visually summarizes the gene expression profiles in IFN-γ+/IL-10+ (yellow), IFN-γ(-)/IL-10+ (violet) and IFN-γ+/IL-10(-) (blue) cells from the 4 different donors. The heat map was generated through Affymetrix software setting an FDR of 0.1. Each column represents a specific sorted population (e.g. IFN-γ+/IL-10(-), blue) of one donor and each row represents a specific gene. Each cell represents the expression level of a single transcript in a single sample. The blue/yellow colour-coded heat map represents relative gene levels with yellow indicating transcript levels above the median for that gene across all samples and blue indicating transcript levels below the median for that gene across all samples. Colour saturation is proportional to the scale of difference from the median. The rows relative to IFN-γ, IL-10 and asparagine endopeptidase (AEP) genes have been highlighted through white boxes. The hierarchical clustering tree shown on the left of the heat map is an agglomerative approach in which gene profiles are joined in groups which are further linked in groups until a single hierarchical tree has been formed. The tree is built through distance matrixes which are calculated by the Affymetrix software to cluster the most similar genes or clusters.

4.4 AEP expression in human CD46 induced T cells

The gene array study conducted using the three populations IFN-γ+, IFN-γ+/IL-10+ and IL-10+ derived from CD3/CD46-activated primary human $CD4^+$ T cells showed significant down regulation in the IFN-γ+ population of the mRNA encoding for the asparagine endopeptidase (AEP) when IFN-γ+/IL-10(-) cells were compared to IFN-γ(-)/IL-10+. To confirm the results obtained from the gene array with assessment of AEP mRNA induction, $CD4^+$ T cells purified from 2 different donors were activated with mAbs to CD3 and CD46 in the presence of 50 U/ml of rhIL-2. The IFN-γ+/IL-10(-) and IFN-γ(-)/IL-10+ cells were FACS sorted and the mRNA was extracted from each population. Real-time PCR was then performed on the reverse transcribed RNA (Figure 4.5a). AEP gene expression was confirmed to be up-regulated in the IFN-γ(-)/IL-10+ cells induced by CD46 activation as previously detected in the gene array analyses. To determine whether AEP was detectable on a protein level, western blot analyses were performed on the nuclear and cytoplasmic fractions of purified $CD4^+$ T cells. T cells activated for 36 hours with mAbs to CD3 showed higher AEP protein levels in comparison to CD3 plus CD46 activation (Figure 4.5b). Moreover, AEP protein levels were mostly up-regulated by activation conditions including mAbs to CD3 plus CD28. The down-regulation of protein levels of AEP upon CD46 engagement during T cell activation may be due to the induction of a higher turnover of the enzyme upon such signals. It is important to consider that the western blot experiment was carried out using whole $CD4^+$ T cells and that the difference on gene level expression highlighted by the gene array refers to the comparison of sorted IFN-γ+/IL-10(-) versus IFN-γ(-)/IL-10+ cells induced by CD46. Hence, although the western blot analysis confirmed AEP expression in activated T cells, the modulation of gene expression shown in the gene arrays was not confirmed on the protein level. To investigate AEP cellular localisation, $CD4^+$ T cells were activated with antibodies to CD3 and CD28 or CD3 and CD46 in the presence of 50 U/ml for 12 hours. Cells were then harvested and stained with antibodies for AEP and LAMP-1 (lysosomal marker). Interestingly, and in accordance with the Western blot data, AEP expression was upregulated upon TCR signals (Figure 4.6). The activation through CD3 and CD46 showed AEP lysosomal colocalisation specifically in blasting cells.

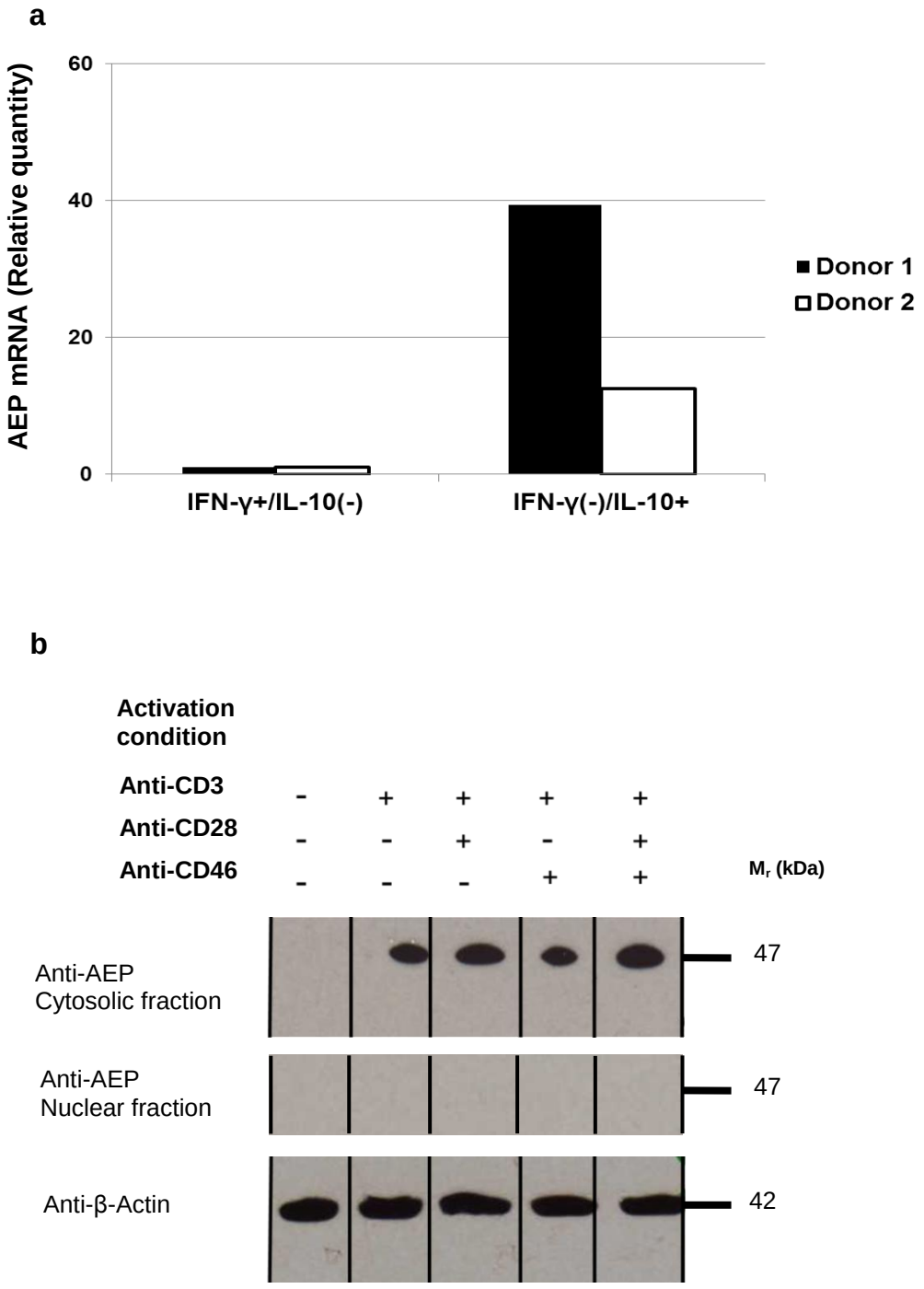


Figure 4.5 AEP mRNA and protein levels in CD46 activated T cells. a) $CD4^+$ T cells were purified from 2 different healthy donors and activated with mAbs to CD3 and CD46 in the presence of 50 U/ml of rhIL-2 for 36 hours. Post stimulation the induced IFN-γ+/IL-10(-) and IFN-γ(-)/IL-10+ populations were sorted by FACS. Subsequently mRNA was extracted and reversed transcribed to cDNA. AEP gene expression was assessed by qPCR normalised to 18S expression (as described in 2.12.5) and is shown as a relative quantity of fold change setting the reference level of expression to the IFN-γ(-)/IL-10+ sample. Data are representative of one experiment including 2 donors. b) $CD4^+$ T cells were purified from healthy donors and activated

with immobilised mAbs to CD3, CD28 and CD46 or left untreated in the presence of 50 U/ml of rhIL-2. Protein extraction was carried out from cytosolic and nuclear fractions after 36 hours of activation. Protein amounts from the different stimulation conditions were normalized using the Bradford assay and were confirmed by Western blot of cytosolic fractions with anti-β-actin mAbs. Nuclear and cytosolic fractions were screened for AEP expression using a polyclonal anti-AEP antibody.

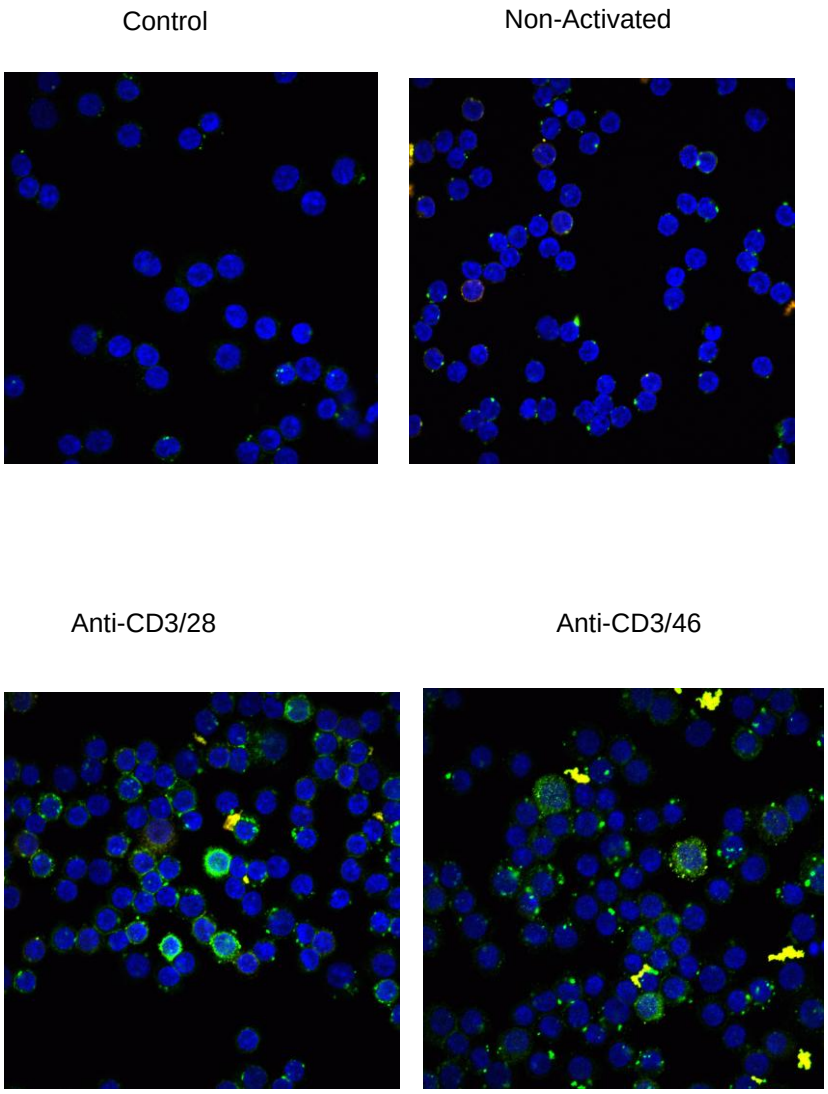


Figure 4.6 AEP localisation in CD4$^+$ T cells. Confocal microscopy of CD4$^+$ T cells left untreated (upper left panel) or activated with mAbs to CD3 and CD28 (lower right panel) or to CD3 and CD46 (lower left panel) in the presence of 50 U/ml of rhIL-2 for 12 hours. Cells were stained for DAPI (blue; nuclear die), LAMP-1 (green; lysosomal marker) and AEP (red) and isotype controls (upper left panel) were included by adding secondary antibodies only (control). Yellow areas indicate co-localization of LAMP-1 and AEP. Pictures (100X Zoom) were taken on an A1R Si Confocal microscope (Nikon, Japan).

4.5 Functional inhibition of AEP in human $CD4^+$ T cells

Having confirmed the expression of AEP in human $CD4^+$ T cells and its modulation by CD46 activation, the effect of AEP inhibition on T cell viability, activation and cytokine production/regulation was next assessed. In the following experiments, a chemical AEP inhibitor (AEPi) developed by Medivir (Stockholm, Sweden) (Figure 4.6a) which has been shown to be highly specific for AEP in several sets of data (Costantino et al., 2008; Loak et al., 2003; Maehr et al., 2005; Manoury et al., 2003; Sepulveda et al., 2009) was used to test the functional significance of AEP inhibition in the T_H1 switch described in Chapter 3. Since the AEPi is soluble in Dimethyl sulfoxide (DMSO) (Costantino et al., 2008; Loak et al., 2003; Maehr et al., 2005; Manoury et al., 2003; Sepulveda et al., 2009) control analyses were performed to assess whether DMSO alone as carrier could affect cellular viability in T cell cultures. T cells left unstimulated or activated through mAbs to CD3, CD28 and/or CD46 were treated with different concentrations of DMSO for 36 hours and analysed for cell viability through a colourimetric assay (Figure 4.6b). Although, no statistical difference was found in any of the conditions between the highest and the lowest concentrations of DMSO, the functional experiments were performed using the lowest concentration of DMSO (1/1000=0.1%). Viability assays were then performed using T cells isolated from four different donors to evaluate the effect of the AEPi on T cell viability. Several functional studies have used the AEPi in concentrations ranging from 20 to 50μM and these concentrations were therefore used as a guide (Costantino et al., 2008; Loak et al., 2003; Maehr et al., 2005; Manoury et al., 2003; Sepulveda et al., 2009). Treating CD3 activated or CD3 and CD46 activated T cells with AEPi at concentrations ranging from 5 to 50 μM had no effect on cellular viability (Figure 4.6c). However, the lack of a positive control for cell death in the viability assays conducted in figure 4.6 does not allow to determine the extent of cell death in the cultures. Inclusion of a positive control for cell death in the assay would have shown the range of absorbance measures associated with cell death which could have then been compared to the observed values for each activation condition.

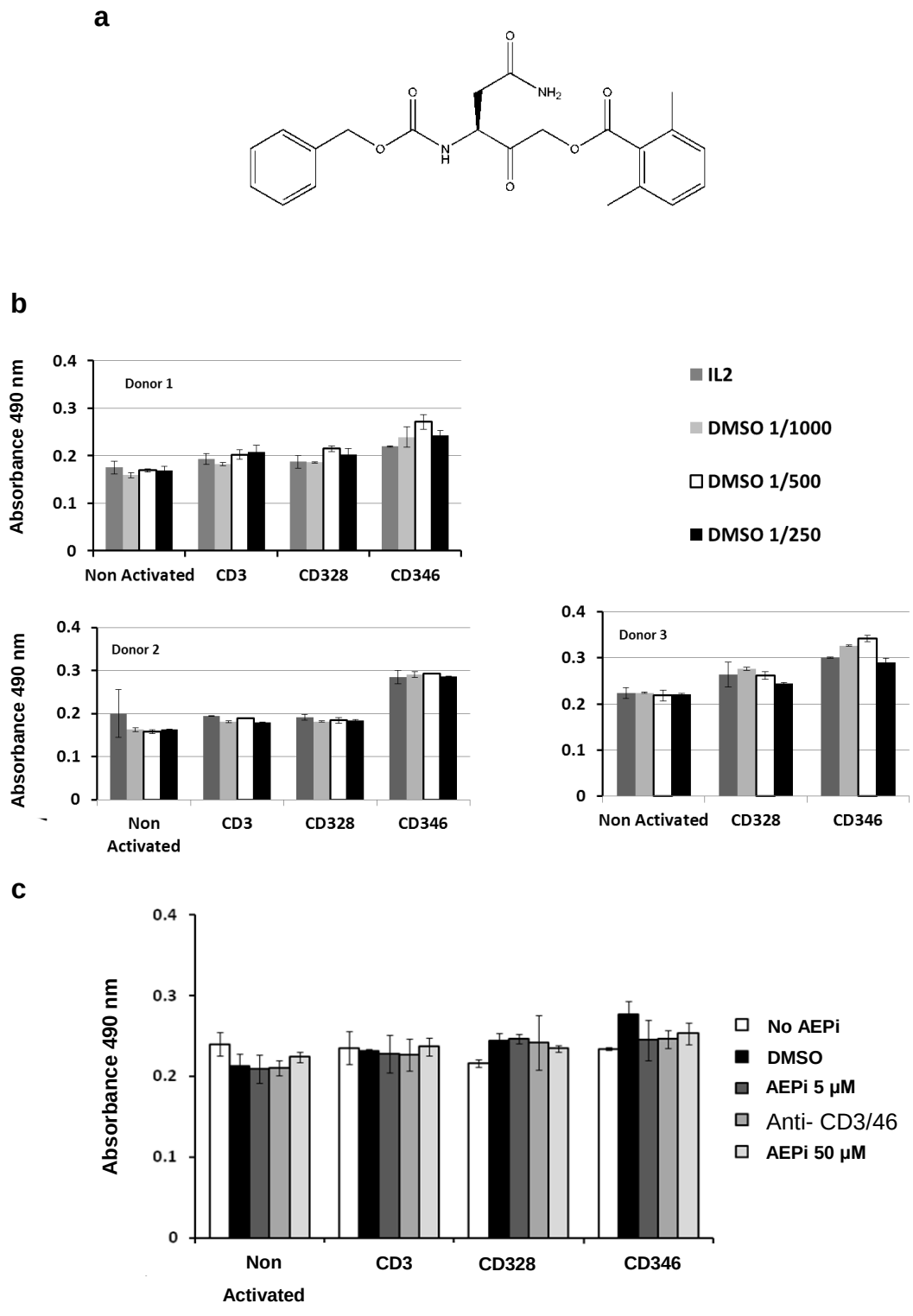


Figure 4.7 Cell viability of AEPi treated CD4$^+$ T cells. a) Chemical structure of AEPi. b) CD4$^+$ T cells were purified from peripheral blood of three healthy donors and activated with mAbs to CD3, CD28 and CD46 in the presence of 50 U/ml of rhIL-2. CD4$^+$ T cells were activated in the presence of increasing concentrations of DMSO (1/1000=0.1%; 1/500=0.2%; 1/250=0.4%). Cellular viability was evaluated using the colourimetric assay CellTiter 96 Aqueous One Solution Cell Proliferation Assay from

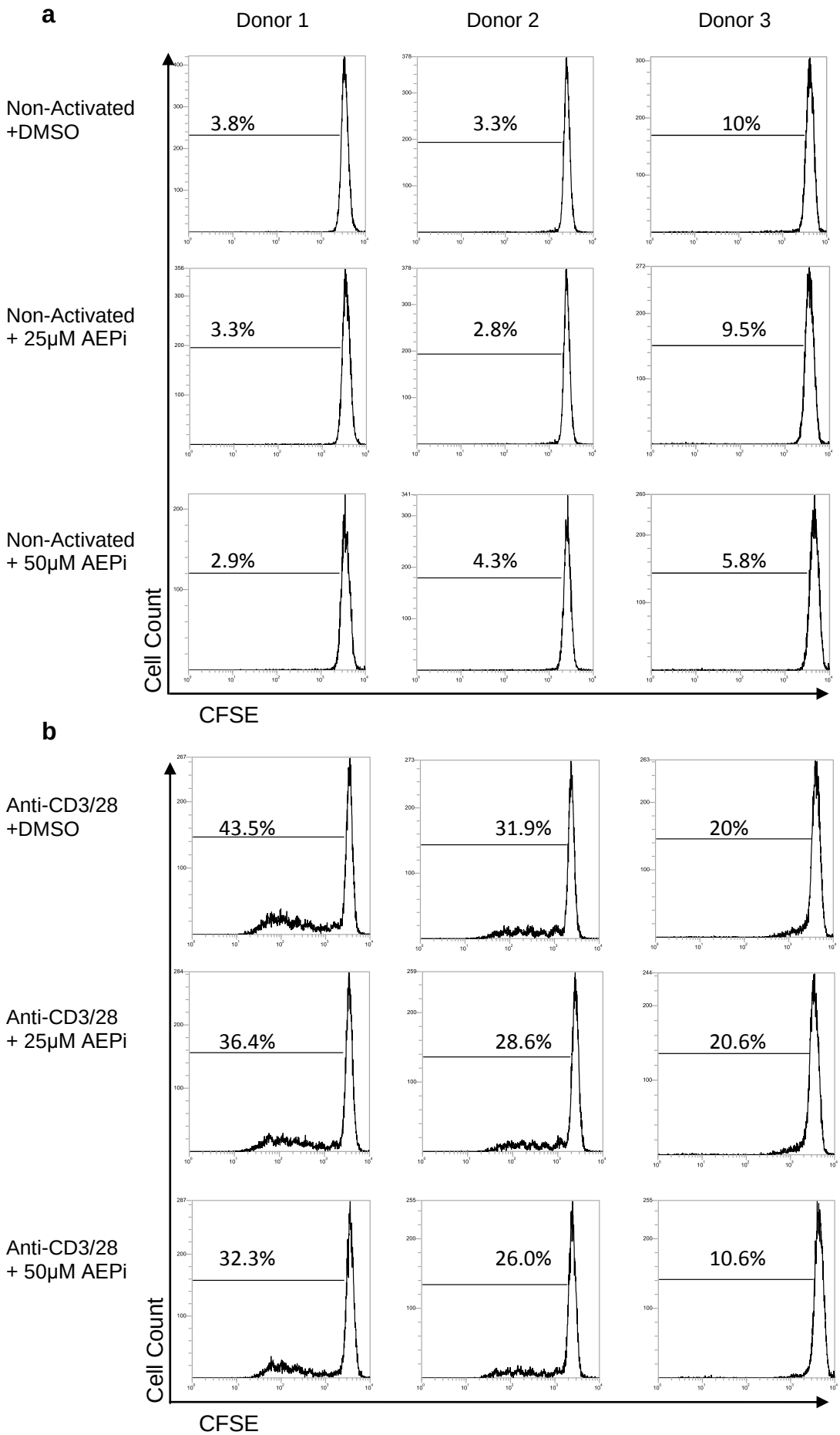
Promega (paragraph 2.8.1). c) AEPi, solubilised in DMSO 0.1%, was added to cultures of activated $CD4^+$ T cells in increasing concentrations (0 to 50 μM). Cellular viability was evaluated as in (b). Data represent results ± SD of 4 separate experiments.

4.6 Functional analysis of AEP inhibition on T cell proliferation and activation

Having established a range of AEPi concentrations that had no effect on cell viability, the effects of AEP inhibition on cellular proliferation were assessed by analyses of CFSE labelled T cells either left non-activated or activated with anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD46 antibodies in the presence or absence of the AEPi (Figure 4.8). AEP inhibition in unstimulated T cells did not evidently decrease or increase cellular proliferation.

The effect on cellular proliferation on AEPi treated T cells activated with mAbs to CD3 plus CD28 (Figure 4.8b) or CD3 plus CD46 (Figure 4.8c) showed a similar trend in these two conditions; in fact proliferation was either not affected or marginally affected when cells were cultured in the presence of AEPi at a concentration of 25 μM. When the AEPi was used at a concentration of 50 μM, it decreased cellular proliferation in both activation conditions. In particular, AEP inhibition at 50 μM decreased cellular proliferation in anti-CD3/CD46 activated cells determining 15% less proliferation in donor 1, 25% less proliferation in donor 2 and 48% less proliferation in donor 3. Anti-CD3 plus anti-CD28 activated T cells cultured in the presence of AEPi at 50 μM decreased proliferation of 26%, 17% and 50% in donor 1, donor 2 and donor 3 respectively.

Of note is that AEPi at a 50 μM concentration had no effect on cell viability (figure 4.7). Thus, AEPi at 50 μM seems to specifically impact on cell proliferation. Although experiments were conducted with both concentrations of the inhibitor it is important to consider that functional implications of AEP inhibition may be due to decreased proliferation when considering the 50 μM concentration. Hence, the 25 μM concentration is more indicative of functional consequences that may be due to the inhibition of AEP activity.


a
Donor 1
Donor 2
Donor 3
Non-Activated +DMSO
Non-Activated + 25μM AEPi
Non-Activated + 50μM AEPi
3.8%
3.3%
10%
3.3%
2.8%
9.5%
2.9%
4.3%
5.8%
Cell Count
CFSE
b
Anti-CD3/28 +DMSO
Anti-CD3/28 + 25μM AEPi
Anti-CD3/28 + 50μM AEPi
43.5%
31.9%
20%
36.4%
28.6%
20.6%
32.3%
26.0%
10.6%
Cell Count
CFSE

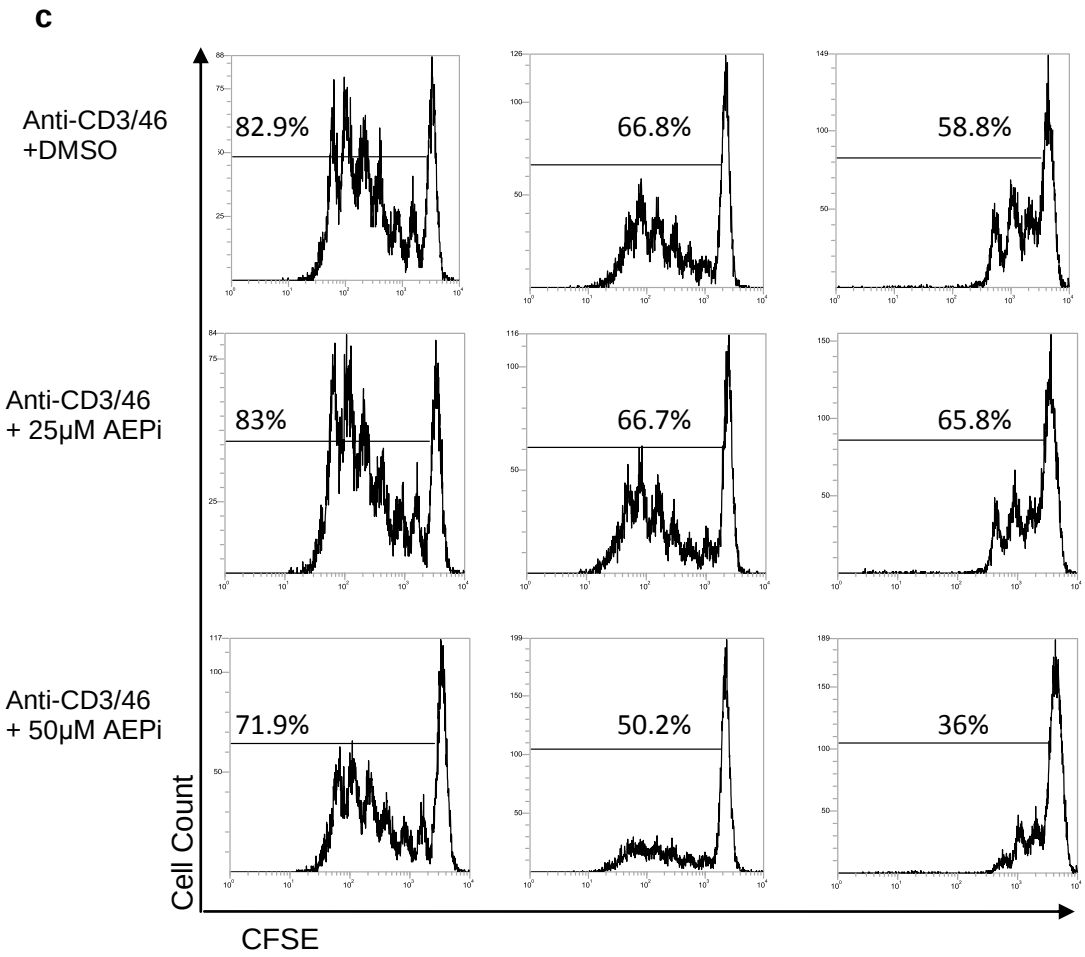
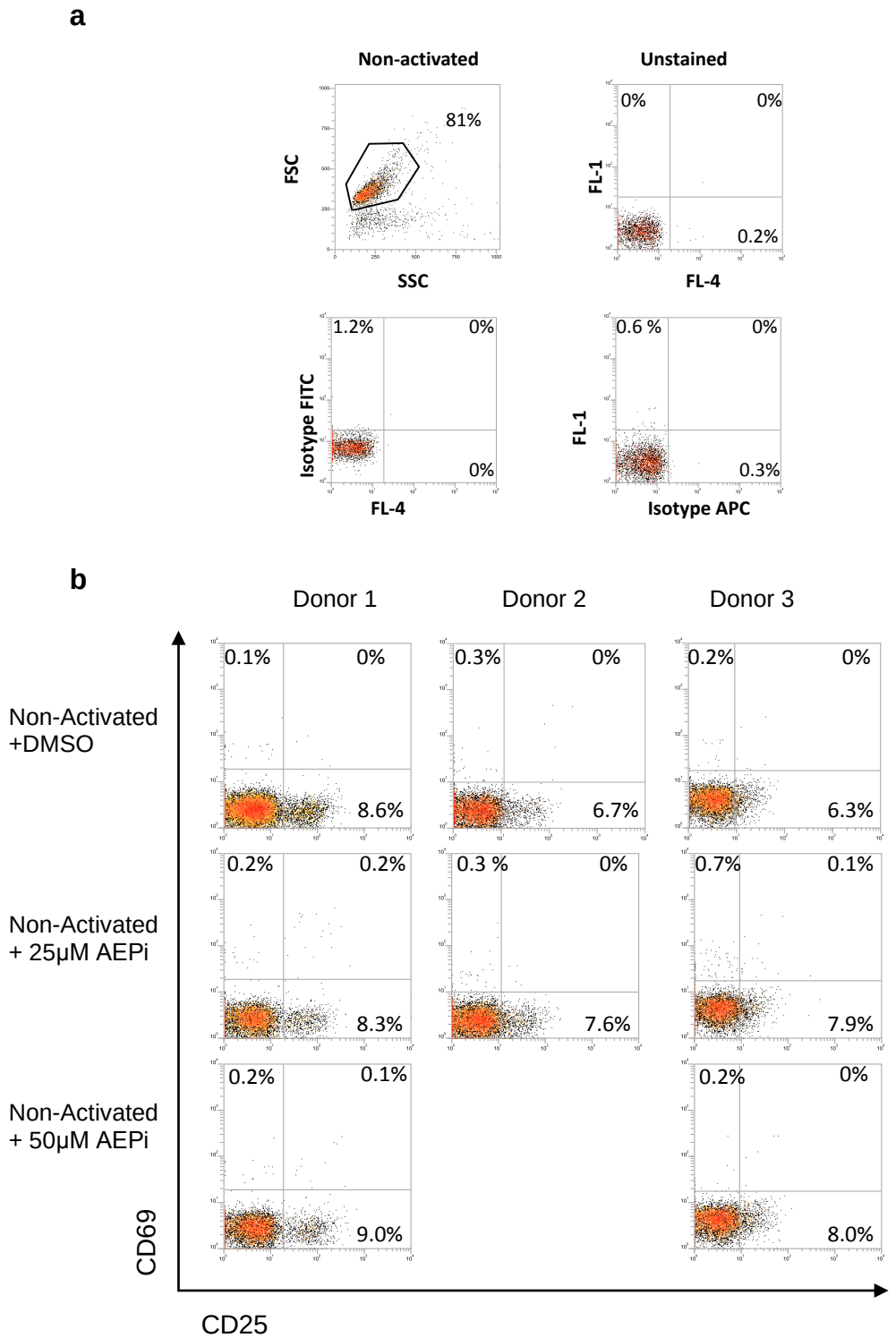


Figure 4.8 T cell proliferation in human AEPi treated CD4$^+$ T cells. CD4$^+$ T cells purified via magnetic cell sorting (positive selection) from peripheral blood of 3 healthy donors were labelled with CFSE (paragraph 2.9.3) and either left unstimulated (a, Non-Activated) or activated with mAbs to CD3 plus CD28 (b) or CD3 plus CD46 (c) in the presence or absence of DMSO only or DMSO solubilised AEPi at a concentration of 25 μM or 50 μM. Non-Activated and activated T cells were cultured for 5 days prior to FACS analysis of CFSE dilution (i.e. proliferation). Values shown are indicative of the percentage of CFSE positive cells within the gate of each histogram.

T cell activation was next assessed by analysing the pattern of expression of the T cell activation markers CD25 and CD69 in activated T cells of 3 different healthy donors (Figure 4.9). Comparison of anti-CD3 and anti-CD46 activated $CD4^+$ T cells in the presence of the AEPi carrier only (i.e. DMSO) or of the AEPi at a concentration of 25 or 50 μM showed no evident difference in the activation marker expression in the three donors (Figure 4.9d).



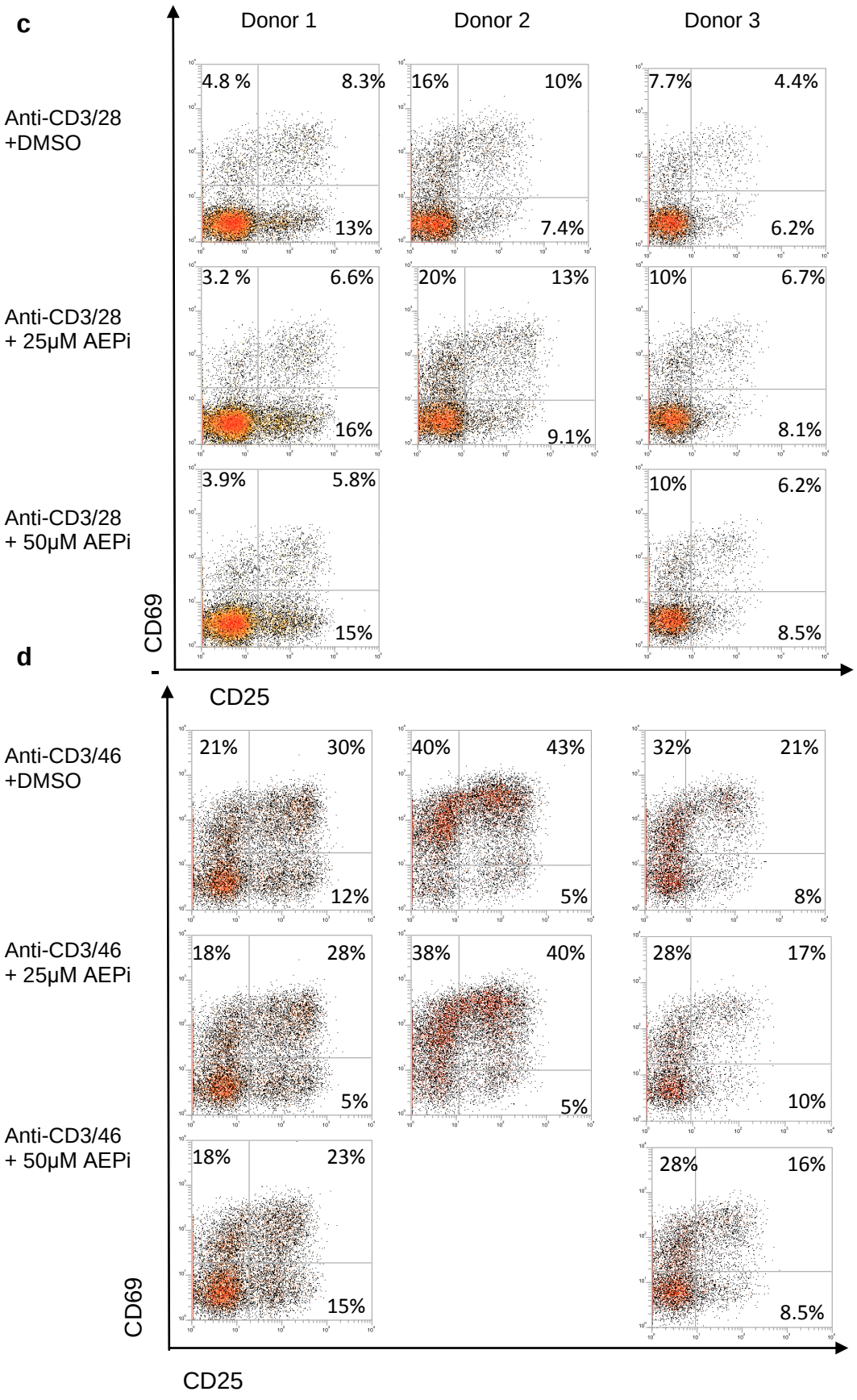


Figure 4.9 T cell activation status in human AEPi treated T cells. $CD4^+$ T cells were magnetically sorted via positive selection from peripheral blood of 3 healthy donors and were either left non-activated or activated with mAbs to CD3 and CD28 or

CD3 and CD46 (2 μg/ml) in the presence of 50 U/ml of rhIL-2 for 36 hours. Unstimulated or activated T cells were cultured in the presence of DMSO or DMSO solubilised AEPi at a concentration of 25 μM (b, c and d; donors 1, 2 and 3) or 50 μM (b,c and d; donors 1 and 3). a) FSC/SSC gating strategy of non-activated cells, unstained control sample (top dot plots), isotype control staining for IgG1-FITC and IgG1-APC (lower dot plots). CD25 (FITC) and CD69 (APC) surface staining of unstimulated (b), anti-CD3 plus anti-CD28 (c) and anti-CD3 plus anti-CD46 (d) activated T cells. Values are indicative of the percentage of positive cells within the relevant quadrant.

4.7 Effect of AEPi on cytokine synthesis and secretion

To determine whether AEP inhibition could affect the CD46-mediated induction of the T_H1 switch, the AEPi was added to cultures of activated $CD4^+$ T cells. Because AEP was identified as the most differentially expressed gene between IFN-γ+ and IL-10+ T cells (Figure 4.3), it was anticipated that inhibition of AEP may impair IL-10 production upon CD3/CD46-activation. First a secretion assay for IFN-γ and IL-10 was performed to evaluate whether AEPi treatment could affect the secretory ability of T cells activated with mAbs to CD3, CD3 and CD28 or CD3 and CD46. Although AEP inhibition affected IL-10 secretion at 25 μM of AEPi the maximal decrease in IL-10 secretion was obtained by adding AEPi at a concentration of 50 μM (Figure 4.10). Importantly the secretion of IFN-γ was also affected showing a negative effect of AEP inhibition not exclusively on IL-10 but also on IFN-γ secretion. A trend was observed in the suppression of cytokine secretion from CD3 and CD3/CD28-activated T cells; however, as expected, AEPi was mostly effective on CD3/CD46-activated T cells. To further corroborate this initial finding, the supernatants from AEPi-treated T cells were collected after 36 hours of activation to measure secreted IFN-γ and IL-10 (Figure 4.10). The CBA analysis of the supernatants was fully in line with the initial secretion assay and showed that adding AEPi had an inhibitory effect on the overall secreted IFN-γ and IL-10 determining circa 50% less secreted cytokine in comparison to control DMSO treated T cells. Further, as shown in Chapter 3 (Figure 3.8), inhibition of the T_H1 phase translates into a limitation of the switch into the IFN-γ(-)/IL-10+ phase. This finding was also verified here through the effect of AEPi (Figure 4.10 and 11); in fact AEP inhibition led to less IFN-γ production and consequently to less IL-10 secretion. This data rather indicate that AEP is required for initial IFN-γ production, and not only for IL-10 switching.

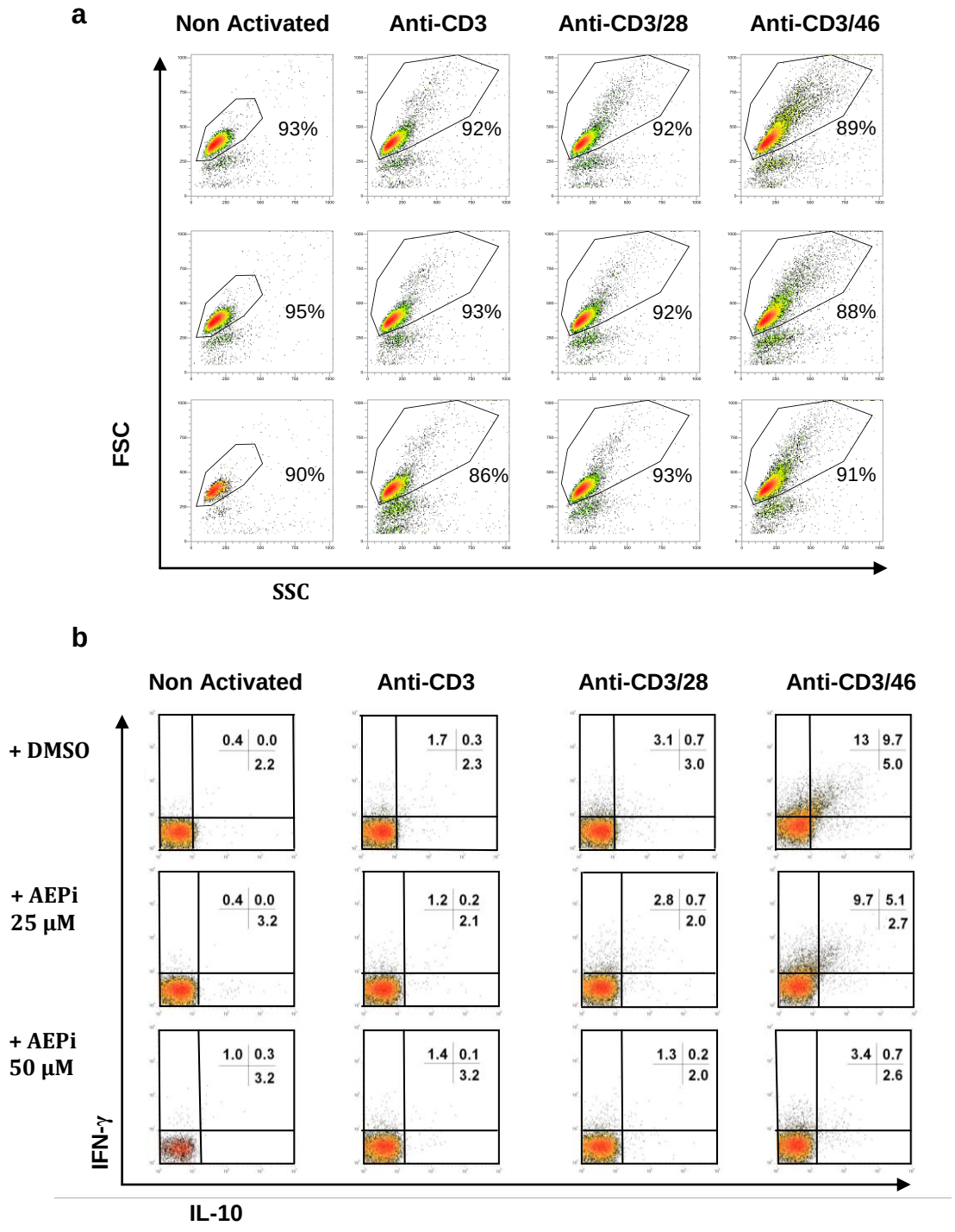


Figure 4.10 Effect of AEPi on IFN-γ and IL-10 secretion in CD46-induced T cells. $CD4^+$ T cells were either left non-activated or activated with plate bound mAbs to CD3, CD28 and/or CD46 (2 µg/ml) in the presence of 25 U/ml of rhIL-2. T cells were either treated with DMSO only or AEPi at a concentration of 25 µM or 50 µM. Following 36 hours of activation secretion assays were performed to identify the IFN-γ (FITC) and IL-10 (APC) secreting T cells a) FSC/SSC gating strategy of non-activated and activated T cells. b) Percentages of IFN-γ and IL-10 secreting T cells relative to

the indicated activating conditions. Values are indicative of the percentage of positive cells within the relevant quadrant.

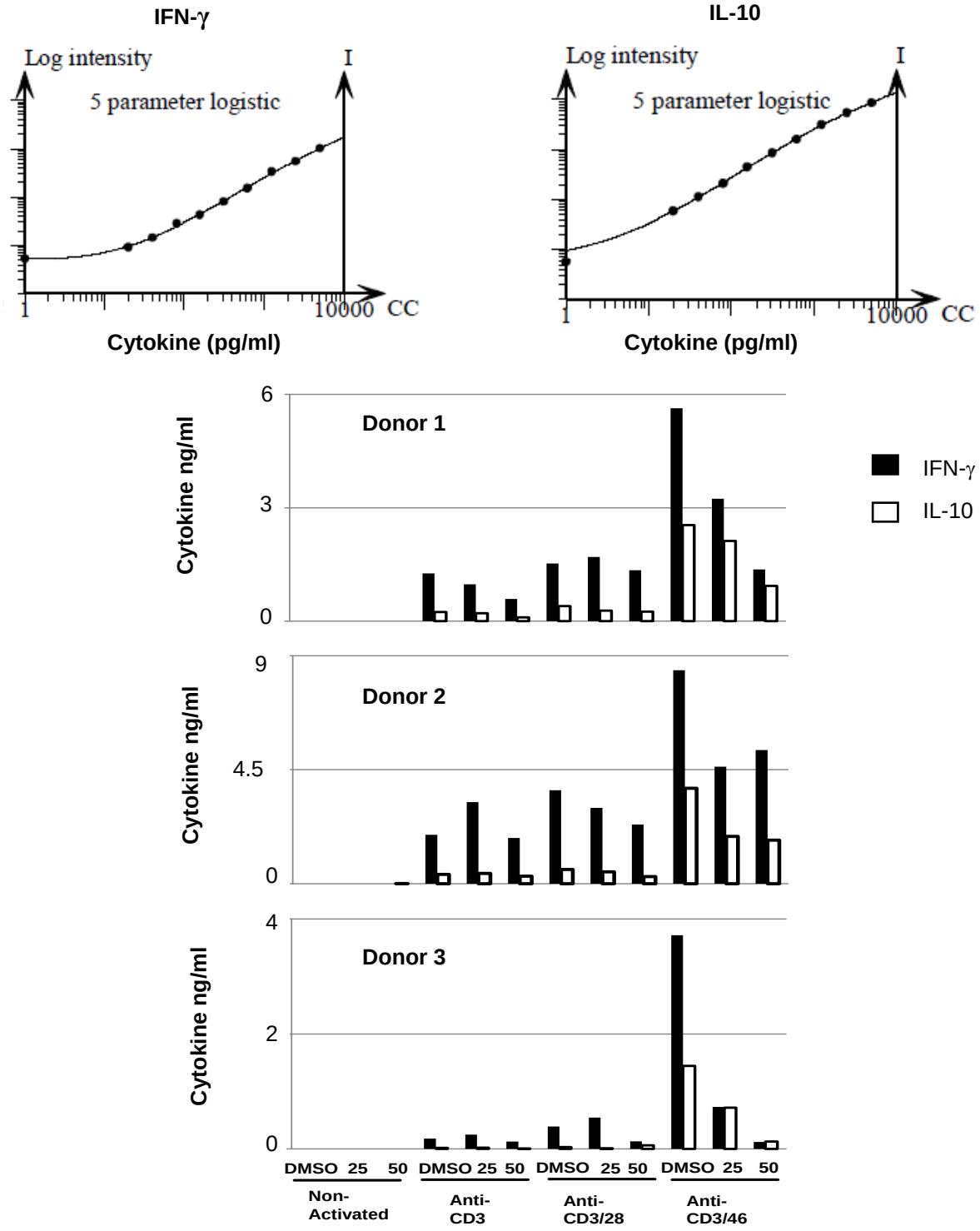


Figure 4.11 AEPi effects on IFN-γ and IL-10 secretion in CD46-induced T cells. Purified human $CD4^+$ T cells were either left unstimulated (non-activated) or activated with plate bound mAbs to CD3, CD28 and/or CD46 (2 μg/ml) in a 48 well plate. Cells were seeded at a concentration of 2.5 x 10^5 per well. Each activation was performed in

the presence of 25 U/ml rhIL-2. Cultures were treated with DMSO only or AEPi at a concentration of 25 μM or 50 μM. Following 36 hours of activation supernatants were harvested to evaluate the amounts of secreted IFN-γ (black bars) and IL-10 (white bars). a) CBA standard curves for IFN-γ and IL-10 quantification expressed in pg/ml. b) IFN-γ and IL-10 quantification in non-activated (NA), anti-CD3, anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD46 activated samples.

Because AEP is also involved in the activation of lysosomal pathways, the mechanism through which AEP may affect IFN-γ production was assessed. Intracellular staining for IFN-γ and IL-10 were performed (Figure 4.12) on $CD4^+$ T cells activated with DMSO or AEPi at 25 and 50 μM. These experiments revealed that the frequency of IFN-γ+ T cells containing this cytokine intracellularly is only marginally affected with 1-3% less IFN-γ+ T cells between DMSO *versus* AEPi treated cells. However, the transition of IFN-γ+ to IFN-γ+/IL-10+ T cells is highly affected with about 30 to 50% less double positive cells in AEPi treated cultures. Hence, AEPi treatment does not block IFN-γ expression *per se* but rather inhibits the secretion of this cytokine by activated T cells. It is being currently assessed whether IFN-γ is produced upon CD3/CD46-actvation in the presence of AEPi on the mRNA level using RT-PCR analyses.

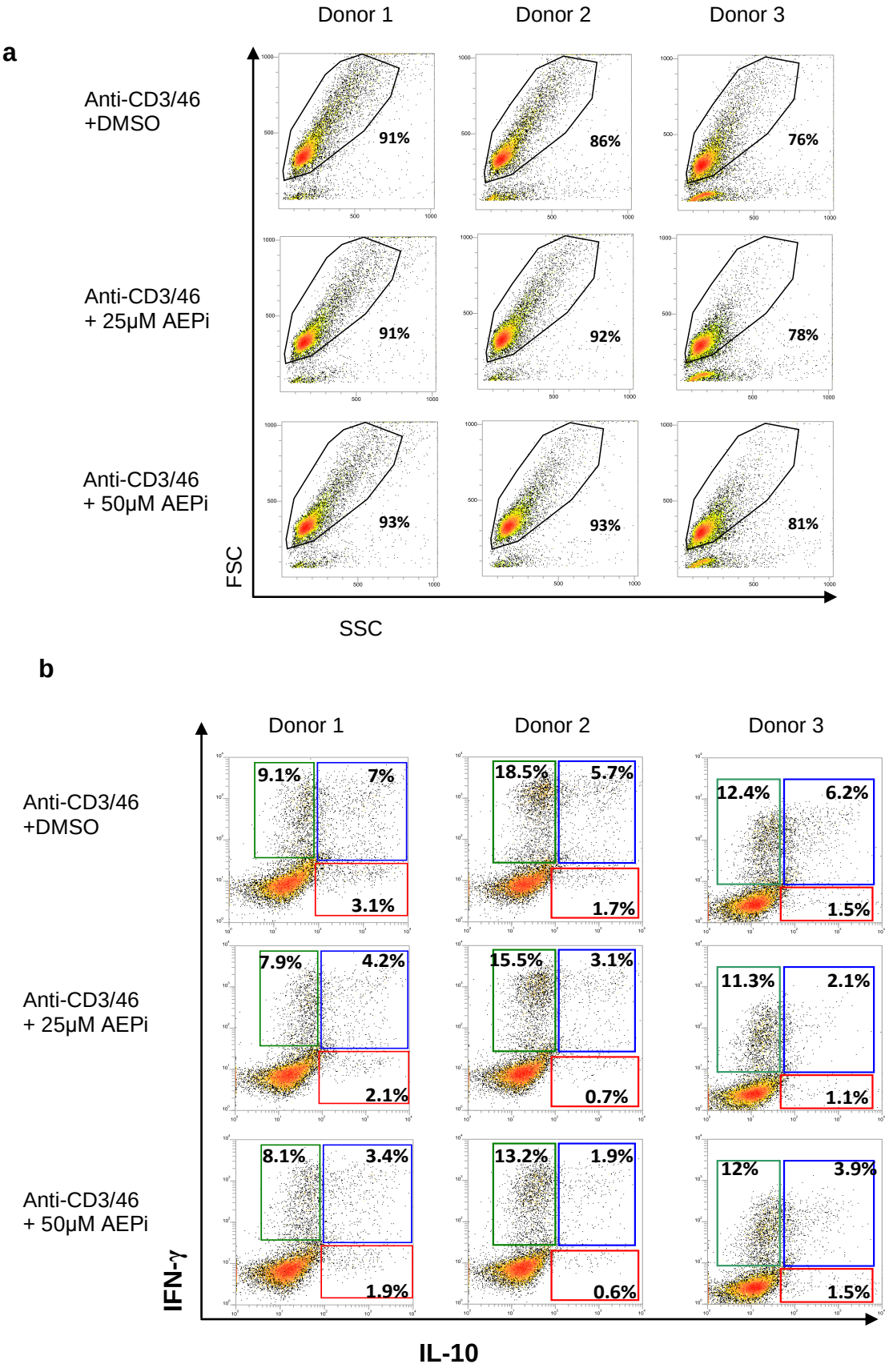


Figure 4.12 AEPi effects on IFN-γ secretion and IL-10 synthesis in CD46-activated T cells. $CD4^+$ T cells were purified via magnetic sorting (positive selection) and activated in the presence of plate-bound mAbs to CD3 plus CD46 (2 µg/ml) in the presence of DMSO or AEPi (25 or 50 µM) and of 25 U/ml of rhIL-2. After 36 hours of

activation, T cells were treated with PMA/Ionomycin for 4 hours and monensin was added to the last 2 hours of culture. T cells were then assessed for intracellular cytokine production by FACS analysis as described in paragraph 2.10.1. a) FSC/SSC gating strategy b) Intracellular staining for IFN-γ (FITC) and IL-10 (APC) in anti-CD3 plus anti-CD46 activated T cells. Values represent the percentage of positive cells within the relevant gate.

4.7.1 Exogenous IFN-γ restores IL-10 production inhibited by AEPi

The previous results demonstrate that AEP inhibition prevents the secretion of IFN-γ but not its production while it clearly reduces IL-10 production. The question that this observation triggered was whether the effect on IL-10 production was a direct effect of AEP inhibition or rather a consequence of the lack of IFN-γ secretion into the micro-environment. To address this question, human $CD4^+$ T cells were activated in the presence of the AEPi with or without the addition of recombinant human IFN-γ; IFN-γ and IL-10 were then measured intracellularly whilst the culture supernatants were only assessed for IL-10 in order to avoid a false positive signal from the added exogenous IFN-γ. In accordance with the previous data (Figure 4.11) addition of AEPi evidently affected IFN-γ+/IL-10+ determining up to 50% less double positive cells in comparison to DMSO treated cultures (Figure 4.13). Importantly, addition of IFN-γ restored both the IFN-γ+/IL-10+ switching population and the synthesis of IL-10 as evaluated by the IL-10 levels in the supernatants of activated T cells (Figure 4.14). These results represent a preliminary set of data derived from one single donor are currently being repeated. However, in conjunction with the data showing that blocking initial T_H1 also results in inhibition of T_H1 switching (Figure 3.8), these findings suggest a direct role for AEP in the initial IFN-γ secretion and an indirect role in IL-10 production.

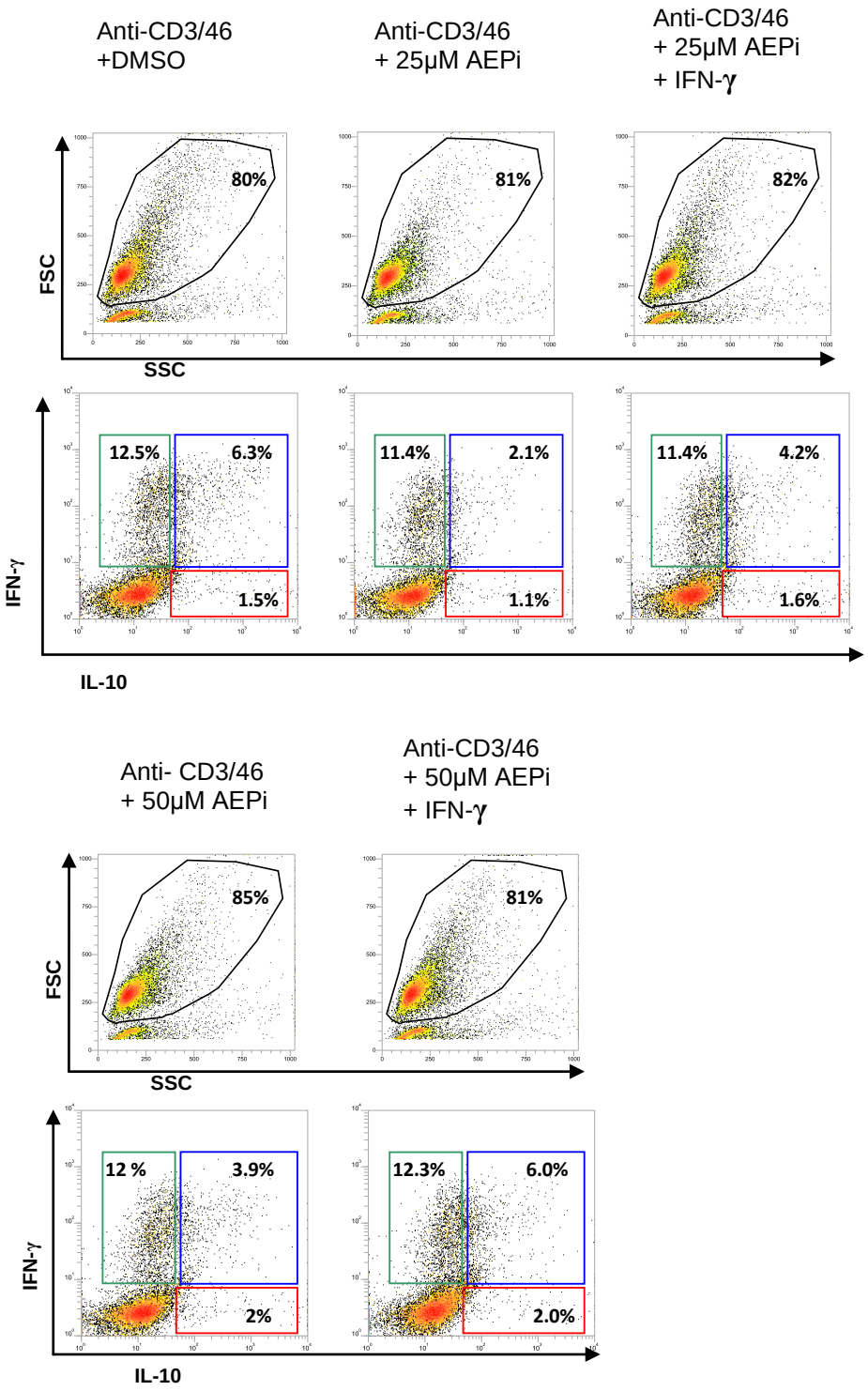


Figure 4.13 Addition of IFN-γ restores the switching of T_H1 cells and the synthesis of IL-10. Purified human $CD4^+$ T cells were left unstimulated (non-activated) or activated with plate bound mAbs to CD3 and CD46 (2 μg/ml) in the presence of 25 U/ml of rhIL-2. Cells were treated with AEPi at 25 μM and 50 μM in the presence or absence of exogenous IFN-γ. IFN-γ was added at a concentration of 10 ng/ml. Cells were seeded at a concentration of 2.5 x 10^5 per well. Following 36 hours of activation T cells were treated with PMA/Ionomycin for 4 hours and monensin was added for last

2 hours of culture. Post stimulation T cells were fixed/permeabilized and stained for intracellular IFN-γ (FITC) and IL-10 (APC).

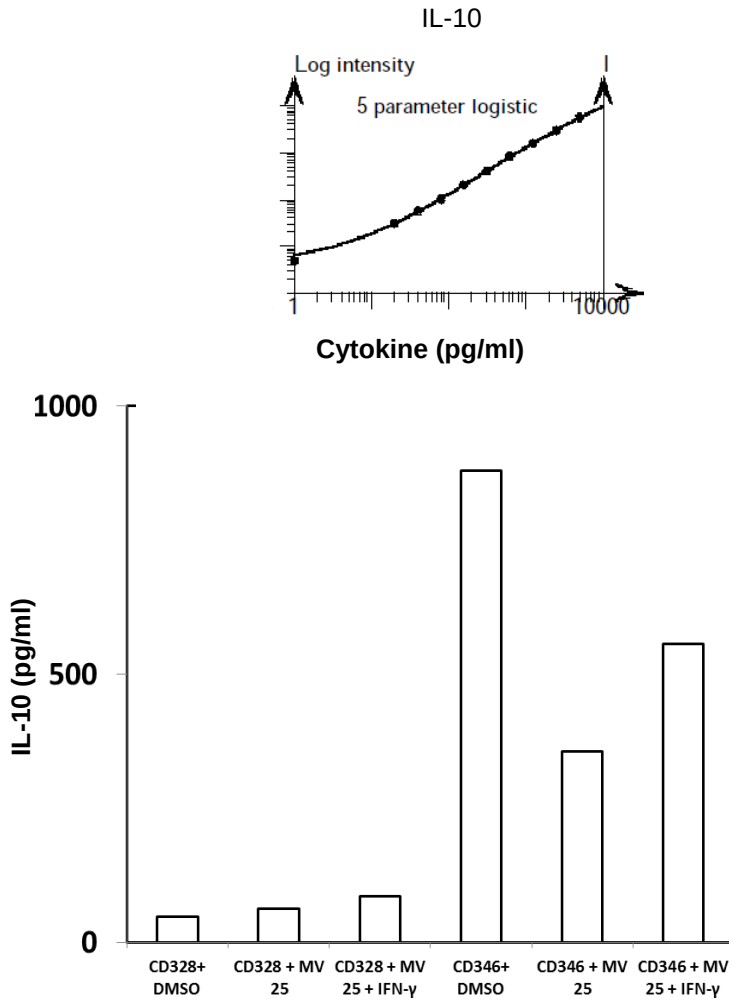


Figure 4.14 Addition of IFN-γ restores the production and secretion of IL-10. $CD4^+$ T cells were activated as described in figure 4.13. Post stimulation (36 hours) supernatants were harvested and screened for secreted IL-10 through CBA. The top panel represents the CBA standard curve for IL-10 quantification, expressed in pg/ml. The lower panel reports the amounts of IL-10 (pg/ml) in anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD46 activated T cells +/- 25 μM of the AEPi and in the absence or presence of exogenous IFN-γ (10 ng/ml).

4.8 Discussion

To characterize the molecular switch from the effector (IFN-γ +) to the regulatory (IL-10+) phase of T_H1-Tr1 switching, gene arrays (Affymetrix) were performed using RNA isolated from blood-purified $CD4^+$ T cells of healthy donors either left untreated (Fig.4.2) or CD3/CD46/IL-2 activated for 36 hours and then sub-sorted into the resulting IFN-γ+, IFN-γ+/IL-10+, and IL-10+ populations. Among the most differentially regulated genes was the *AEP* gene, which encodes a lysosomal cysteine protease with multiple immunological functions (Maehr et al., 2005; Manoury et al., 2003; Sepulveda et al., 2009). Quantitative PCR (qPCR) analysis confirmed higher *AEP* gene expression in IL10+ T cells than in IFN-γ+ lymphocytes. Immunoblot analysis confirmed AEP expression in $CD4^+$ T cells with a strong modulation upon TCR signalling. In particular, CD46-mediated signals determined a downregulation of AEP expression over time which is in contrast with data obtained through the gene array and the qPCR; this might be explained by the high turnover of the enzymatic activity of AEP upon CD46 activation. However, a more likely explanation is that the expression analyses were performed so far on bulk-activated $CD4^+$ T cells and not on the sorted populations (IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+). This is currently under way and should then clarify this point more satisfyingly.

The chemical inhibitor developed by Medivir, which is highly specific to AEP (Costantino et al., 2008; Loak et al., 2003; Maehr et al., 2005; Manoury et al., 2003; Sepulveda et al., 2009) was used as a tool to address the hypothesis that AEP is involved in CD46-mediated T cell activation influencing either the inflammatory pathway of IFN-γ, the generation of T_H1 cells and/or the regulation of the T_H1 switch into IL-10. CD46-driven IFN-γ secretion (but not expression) was substantially reduced by AEP inhibition and cells were unable to produce IL-10 and thus 'switch'. However, re-establishing signalling through the IFN-γ receptor by adding exogenous IFN-γ into the cultures restored the generation of the usually observed IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ populations. These data infer an important point: IFN-γ receptor signalling itself seems to be a prerequisite for subsequent IL-10 production. This finding is in line with the suggestion that an effector T cell has to pass through a pro-inflammatory phase before it can enter a regulatory phase, a mechanism that prevents the direct induction of an immunosuppressive phase by pathogens

(Kemper et al., 2011). Mechanistically, this could be achieved by the presence of binding sites within the IL-10 promoter for transcription factors that are activated by pro-inflammatory cytokines. Indeed, the IL-10 promoter contains STAT1 and STAT5 binding sites (Schaefer et al., 2009; Tsuji-Takayama et al., 2008). Although the data relative to the addition of IFN-γ in the presence of the AEPi showed a tendency for the restoration of the IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ populations (Figure 4.13) and of IL-10 secretion (Figure 4.14) in CD46-activated T cells, it does not indicate the molecular mechanism/pathway by which this restoration may take place. Since AEP is a lysosomal enzyme it is likely that targets of AEP's enzymatic activity reside in this cellular organelle and are not processed in the presence of the AEP inhibitor. In the absence of redundant activity for cleavage/maturation of such targets with other lysosomal enzymes, AEP inhibition may drastically reduce the processing of molecules that are important for the synthesis or secretion of IFN-γ. An important target of AEP's enzymatic activity is represented by prothymosin alpha (ProTα) a short peptide of 109 amino acids, highly conserved and found to be abundant in the thymus (Haritos et al., 1985; Sarandeses et al., 2003). As a result of AEP's activity ProTα is cleaved to generate thymosin α1 and thymosin α11, smaller fragments characterised by immunoregulatory functions (Sarandeses et al., 2003). Thymosin α1 has been shown to be able to prime DCs for antifungal T_H1 resistance through TLR/MyD88-dependent signalling conferring *in vivo* protection to Aspergillus (Romani et al., 2004). The mechanisms through which thymosin α1 exerts these functions include up-regulation of TLR2 and TLR9 expression, in both murine and human DCs, and promotion of IL-12 synthesis and secretion. Importantly thymosin α1 has also been shown to promote interferon-α/γ dependent effector pathways through augmentation of T cell proliferation and activation of T cell effector function (Bozza et al., 2007). Hence, it could be postulated that the inhibition of AEP in CD46-induced T cells interferes with the processing of ProTα into thymosin α1 which could in turn decrease T cell activation and the production of cytokines including IFN-γ. Future experiments addressing this point would shed light on whether CD46 signals are linked to increased ProTα processing, thymosin α1 generation and IFN-γ production/secretion. The unexpected finding that AEP is not directly inducing IL-10 production but is rather required for the secretion of IFN-γ (which in turn leads to IL-10 switching) may help explain the seemingly counter-intuitive gene array data shown

in Figure 4.2. The array and real time PCR data clearly show highest mRNA production in the IL10+ and not IFN-γ+ T cells, and activated (non-switched) T cells contain higher amounts of AEP protein (Figure 4.5). Together with the observation that CD46-induced T cells switching towards an IL-10 phase have the ability to revert to a pro-inflammatory IFN-γ programme, the following model can be derived: resting T cells contain already sufficient amounts of AEP to drive quick and efficient IFN-γ secretion upon CD3/CD46-activation. However, up-regulation (or fresh induction) of AEP gene expression may be important to restore these AEP basal levels and set IL-10+ cells again to be reactive towards new incoming inflammatory stimuli which require IFN-γ secretion. This explanation is in line with the role of AEP in the activation of inflammatory receptors such as TLR3, TLR7 and TLR9 (Ewald et al., 2011; Sepulveda et al., 2009). TLRs have been shown to play a pivotal role in the induction of signals in APCs (O'Neill, 2006) and reports of TLR signalling in T-regs have shown that it may increase their regulatory function (TLR5). A recent study identified TLR9 as an important receptor in adaptive IL-10-producing $CD4^+$ T-regs induced by vitamin D. Engagement of TLR9 through its physiological ligand (CpG) in IL-10 secreting T cells induced by vitamin D was able to induce IFN-γ production and loss of their regulatory capacity (Urry et al., 2009). With CD46 being able to induce the switch of IFN- γ/T_H1 cells to IL-10/Tr1 it is tempting to link CD46 signals to TLR9 in CD4+ T cells. In fact the up-regulation of AEP, involved in the cleavage of TLR9 at the level of the lysosomes, may influence T cell receptivity towards TLR9 ligands and tip the balance towards a pro- or anti-inflammatory state. According to the model suggested CD46-derived IL-10+ T cells would have upregulated the AEP gene expression rendering themselves reactive to the presence of pro-inflammatory stimuli such as the ligand for TLR9, CpG.

Future studies including the usage of inhibitors for the lysosomal and secretory pathways should address whether AEP is important for the maturation of secretory lysosomes (Blott et al., 2002) and highlight the role of AEP in T_H1 induction and switching. In order to develop a better understanding of the exact function of AEP in human T_H1 induction and regulation, gene array analyses are being performed in which mRNA profiles of CD3/CD46/IL-2-activated T cells are compared in the presence or absence of the AEPi. The data obtained should deliver vital clues for the AEP targets and downstream pathways required for its function in normal T_H1

induction and then guide future experiments. A second strategy is also currently being adopted to further pinpoint the exact function of AEP in T_H1 cells. The usage of a specific and widely acknowledged AEP inhibitor is helpful but also has disadvantages. It is difficult to conclusively assess how well AEP activity is inhibited in the T cells during activation. Thus, it cannot be gauged how vital AEP is in IFN-γ production or if another protein may be able to partially compensate for AEP inhibition. To address this point, T_H1 cell induction (*via* TCR/CD28 and IL-12) in murine $CD4^+$ T cells from AEP-deficient mice is being compared to WT animals. The reasoning behind this is two-fold: if a similar phenotype is observed in T cells from $AEP^{-/-}$ mice (thus, lack IFN-γ production and secretion and IL-10 switching), a small animal model to further address the novel role of AEP in T_H1 regulation *in vivo* would be available. Secondly, although there would be major differences in the upstream signalling that is related to the CD46 induction of T_H1 cells in humans and the CD46-independent induction of T_H1 mouse cells, AEP might represent a converging point of such pathways and the downstream molecules might be shared between human and mouse T_H1 cells. Hence, $AEP^{-/-}$ animals may possibly prove to become an invaluable tool to analyse T_H1 regulation *in vivo*.

Chapter 5

CD46 is involved in epithelial cell barrier homeostasis

5.1 Introduction

The gene arrays performed using the sub-sorted three populations of the CD3/CD46/IL-2-driven T_H1 life cycle, IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+, also revealed an additional interesting pattern: while the IFN-γ+ T cell population expressed genes connected with pro-inflammatory responses, the switched IL-10-secreting cells were enriched for genes that are commonly connected with extra cellular matrix remodelling (e.g. metalloproteinases, collagens) and receptors responding to clotting cascade activation fragments (e.g. protease-activated receptors, thrombin receptors). These data suggested that T_H1 cells may impact differently on their surrounding tissue depending on which phase of their 'life cycle' they are. Thus while IFN-γ+ T cells may be destructive for tissue as it is commonly observed during pathogen clearance (Gazzinelli et al., 1996; Hunter et al., 1997), the switched IL-10 cells may not only induce T_H1 response contraction but possibly also contribute to the tissue repair required after pathogen clearance. In order to address such crosstalk between CD46-activated T cells and tissue (here epithelial cells were chosen), a suitable model would be the co-culture of epithelial cells and T lymphocytes in a wound healing assay. However, several studies suggest that CD46 can also signal on epithelial cells and may play a role in epithelial cell barrier integrity. Hence, the consequences of CD46 activation on epithelial cells need to be assessed for the future distinction of each cell contribution in the T cell/epithelial cell crosstalk model. For example, CD46 plays a role in the polarization of human epithelium; in fact the tail CYT-1 of CD46 binds to the protein discs large homolog (DLG) 4 in kidney epithelial cells and this interaction is important for cellular polarization (Ludford-Menting et al., 2002). The interaction of CD46 with *Neisseria meningitidis* or *N. gonorrhoeae* (two of the CD46-binding pathogens) also plays an important role in colonization of several human epithelial cells by this pathogen. The CD46/*Neisseria* interaction leads to different epithelial cell modifications including: rearrangement of the cytoskeleton, calcium flux and ultimately bacterial uptake by the epithelial cell (Crimeen-Irwin et al., 2003; Kallstrom et al., 1998; Lee et al., 2002). In addition, data generated and published in Dr Claudia Kemper's laboratory showed that CD46 and the Ste20/SPS1-related serine/threonine kinase (SPAK) interact in human $CD4^+$ T cells and this interaction is required to switch IFN-γ-producing T_H1 cells into the IL-10-secreting self-regulative state (Cardone et al., 2010). SPAK is better known for its function in

osmotic stress regulation (Li et al., 2004; Smith et al., 2008) as well as regulation of intestinal barrier integrity (Yan et al., 2009; Yan et al., 2007). In fact mutations of SPAK in humans are connected with reduced epithelial cell/cell adhesion and onset of IBD (Yan, et al., 2007). Further, the same yeast-two-hybrid screen (performed by Dr Kemper) that indicated SPAK as a potential binding partner also indicated E-cadherin and α-E-catenin as potential binding partners for the intracellular domain(s) of CD46. α-E-catenin and E-cadherin are key constituents of the E-cadherin/catenin network forming adherens junctions that are vitally required for normal cell/cell interactions and epithelial cell layer maintenance (Kobielak and Fuchs, 2004; Van Aken et al., 2001; Vermeulen et al., 1999; Yamada et al., 2005). These observations suggested that CD46-mediated signalling events could communicate with the E-cadherin/catenin network and/or SPAK in intestinal epithelial cells and contribute to epithelial cell barrier integrity. The involvement of CD46 in the regulation of integrity and inter-cellular communication of intestinal epithelial cells has not been investigated before.

5.2 Hypothesis and Aims

The observations reported above led to the hypothesis that CD46-mediated signalling events communicate with the E-cadherin/catenin network and/or with SPAK in intestinal epithelial cells and contribute to epithelial cell barrier integrity. Hence, the main aim of this chapter was to assess whether CD46 interacts with SPAK and/or E-cadherin and α-E-catenin and to evaluate if the CD46-mediated signalling in intestinal epithelial cells is involved in epithelial cell viability, proliferation and wound healing processes.

5.3 CD46 interacts with proteins required for intestinal epithelial cell layer integrity

Several signalling molecules have been described to be involved in signal transduction mediated by the intracellular domains of CD46, CYT-1 and CYT-2 (Astier and Hafler, 2007; Riley-Vargas et al., 2004). Despite this knowledge, the signalling pathways and their functional significance in different cell types are mostly unknown. To first assess whether CD46 is expressed on the intestinal epithelial cell line Caco-2, cells were stained for CD46 and were shown to be positive for CD46 surface expression (Figure 5.1a). Experiments conducted in collaboration with Dr Clauda Kemper were then performed in order to determine whether CD46 binds to SPAK, α-E-catenin or E-cadherin in the human epithelial colorectal adenocarcinoma cell line, Caco-2, cells were either left unstimulated or activated for 3 hours through anti-CD46 mAbs. Following stimulation cells were lysed and immunoprecipitated with an anti-CD46 mAb overnight. Immunoblottings were then performed by Dr Kemper for SPAK, α-E-catenin or E-cadherin (Figure 5.1b). The data showed that SPAK constitutively bound to CD46 in Caco-2 cells, while the E-cadherin/CD46 interaction was only observed when the cells were activated with monoclonal antibodies (mAbs) to CD46. In contrast, coimmunoprecipitation of CD46 and α-E-catenin in Caco-2 cells was not shown.

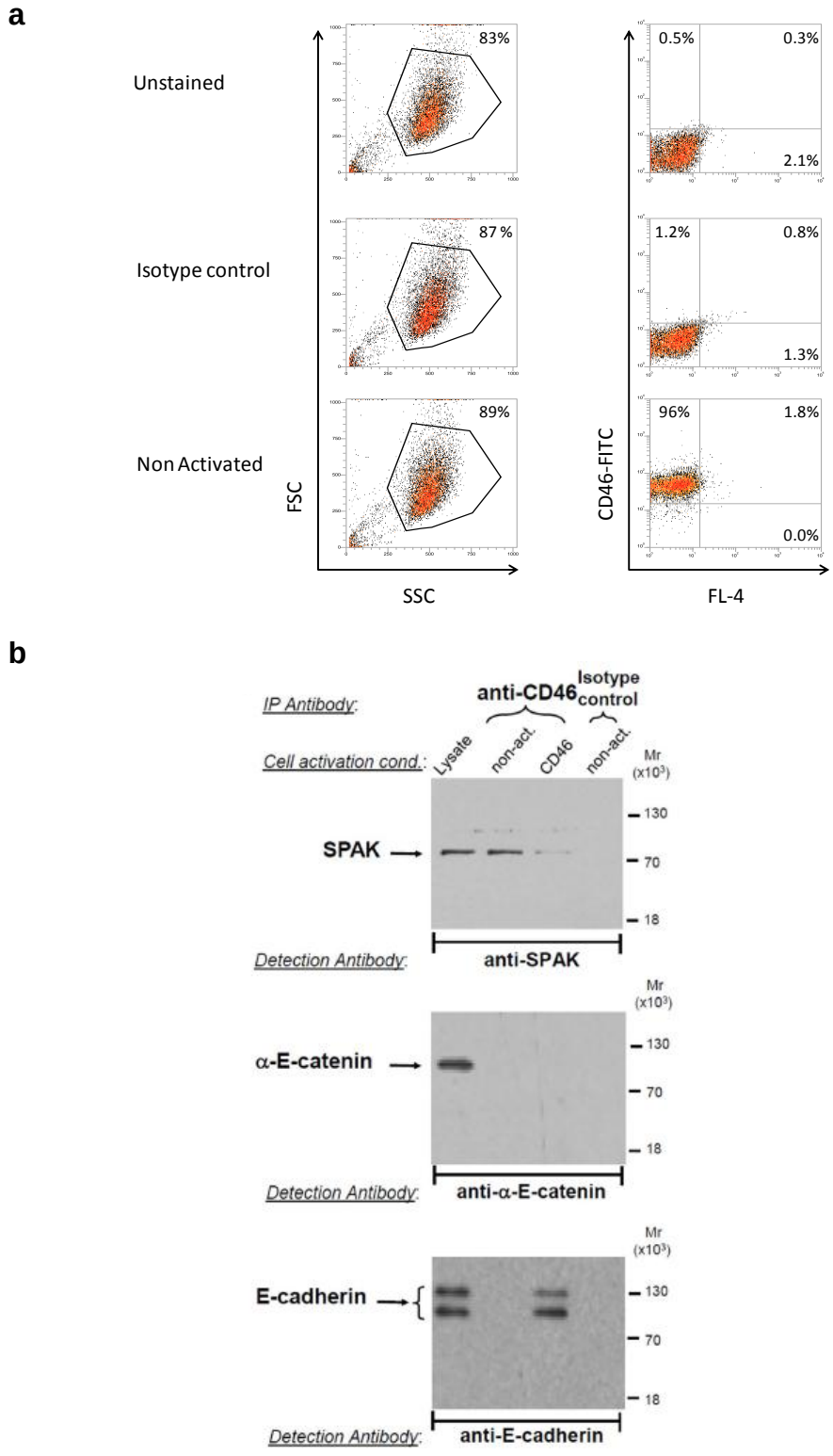


Figure 5.1 CD46 expression in Caco-2 cells and interaction with proteins required for intestinal epithelial cell layer integrity. a) Caco-2 cells were seeded in 48 well plates ($5x10^4$ cells per well) and cultured in the appropriate media without stimulation as described in paragraph 2.2.2. Upon confluence Caco-2 cells were harvested and stained for CD46. Gating strategy for unstained, unstimulated isotype control stained and non-activated CD46 stained Caco-2 cells. Values are indicative of the percentage

of positive cells within the relevant gate or quadrant. b) Caco-2 cells were activated for 3 hours by receptor cross-linking using the antibodies indicated, and cell lysates used for immunoprecipitation with a CD46-specific or isotype control mAb. Immunoblotting with anti-SPAK, anti-α-E-catenin and anti-E-cadherin antibodies was then performed revealing an association between CD46 and SPAK and CD46 and E-cadherin. Untreated lysates from non-activated cells were loaded as positive control ("lysate"). Data are representative of three similarly performed experiments. ^Immunoblots performed by Dr Claudia Kemper.

5.4 CD46 signals impact on epithelial cell barrier function

The functional implications of the interaction between CD46 and SPAK or E-cadherin in Caco-2 cells were next assessed. To determine whether CD46-induced signals can modulate epithelial barrier integrity, a trans-well system was used to test epithelial cell barrier function. The formation of intercellular tight junctions (TJ) and adherens junctions is a rate-limiting barrier, inhibiting paracellular flow of ions and larger solutes resulting in measurable differences in the ion concentration between the upper and lower chamber of the trans-well. These ion concentrations differences are measured and expressed as trans epithelial resistance (TER). Caco-2 cells were cultured for up to 10 days and functional barrier formation through TJ and AJ assembly monitored *via* increasing TER (Figure 5.2a). Monoclonal antibodies to CD46 were added to Caco-2 cultures with a TER $\geq$ 1000 Ω/cm^2. Within 30 min post activation TER decreased by 70% of the initial value while an isotype control mAb showed only marginal TER decrease (Figure 5.2b). Ethanol (EtOH), was added at a concentration of 15% to the cultures as a positive control of barrier disruption and reduced TER to about 60% albeit with slower kinetics compared to CD46 activation (Figure 5.2b). Reduction of TER was maintained in the presence of CD46 activation for up to 36 hours (Figure 5.2b).

The biological function of TER regulation includes epithelial cell growth control and the controlled transport or transmigration of solutes and particles between the paracellular space of epithelial cells via the 'opening' of TJs and AJs. Evidence underpinning that CD46 activation of Caco-2 cells regulates paracellular permeability was provided by the observation that the addition of CD46 cross-linking mAbs to cell cultures increased the transmigration of FITC-labeled 5 kDa dextran beads from the upper to the lower trans-well chamber significantly (Figure 5.2c). Importantly, the kinetics of CD46-induced bead transmigration were fully in line with those observed

for CD46-mediated TER changes (Figure 5.2b). To assess CD46's function in epithelial cell barrier function towards a more physiological 'agent', the transmigration of the uropathogenic *E. coli* strain J96 in the presence of CD46 activation was next analysed (Figure 5.2d). Similarly to the experiments using beads, *E. coli* bacteria accumulated faster and in higher numbers in the lower trans-well chamber when CD46 activation took place. The high statistical deviation observed when bacteria were used in the transmigration experiments is likely due to the more complex interaction of the microbes with the epithelial cells (e.g. concurrent activation of toll-like receptors etc.) compared to the usage of beads. Importantly, the observed CD46-mediated increase in TER and paracellular permeability in Caco-2 cell cultures are not due to increased apoptosis as neither the addition of mAbs to CD46 nor the isotype control mAb increased Annexin V binding (Figure 5.3). Thus, these data indicate that CD46 activation modulates TJ and AD expression or function and by this decreases barrier function of intestinal epithelial cells.

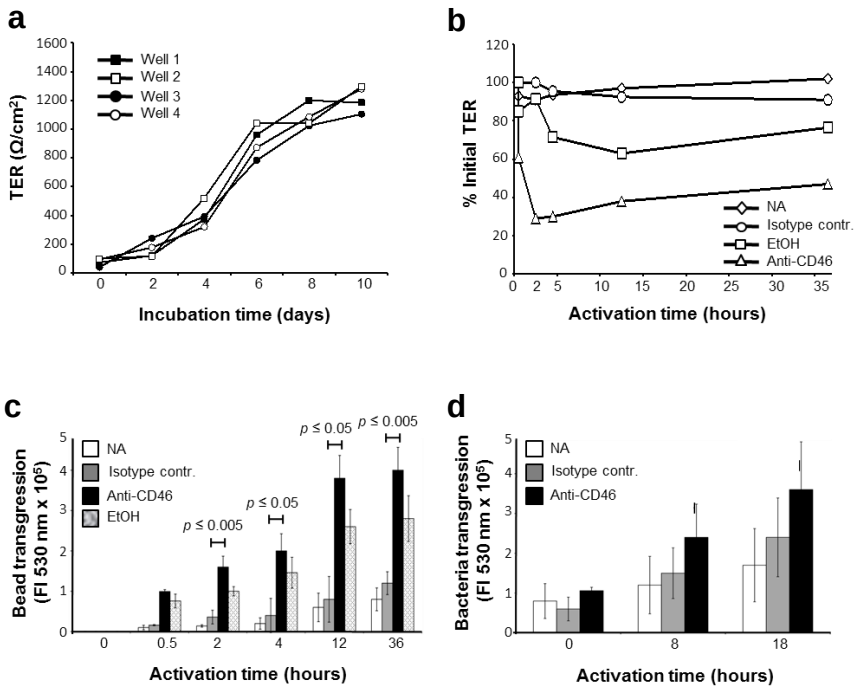
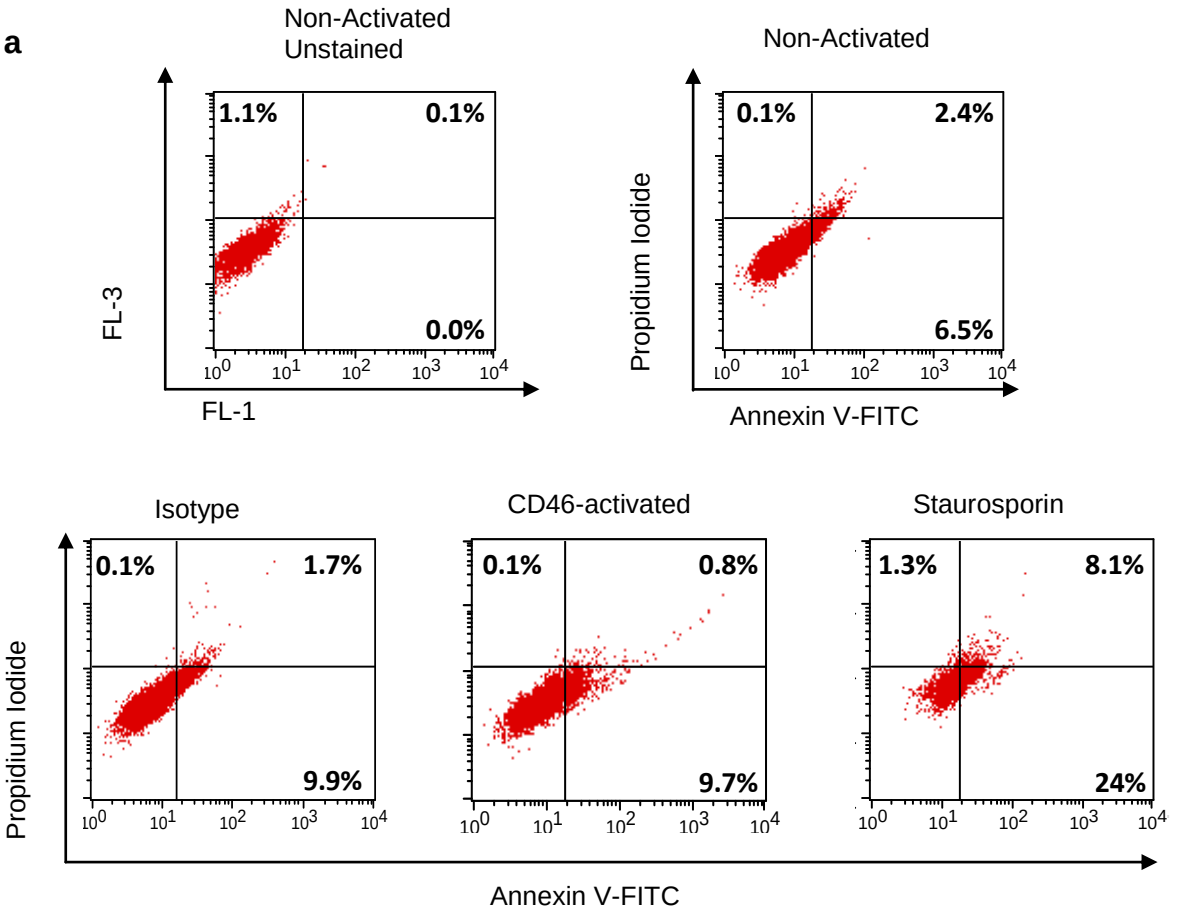


Figure 5.2 CD46 regulates epithelial cell barrier integrity in Caco-2 cell monolayers. (a) Caco-2 cells were seeded at 5 x 10^4 cells/well in the upper well of a trans-well system. Trans-epithelial resistance was assessed every 2 days for a period of 10 days using the EVOM2 voltohmmeter. Shown is the TER measured in 4 wells over 10 days. Data are representative of 3 similarly performed experiments. (b) Caco-2 cells were cultured in trans-wells until a minimum trans-epithelial resistance of 1000 Ω/cm^2 was reached. Subsequently anti-CD46 mAb (10 µg/ml), isotype control mAb (10 µg/ml) or 15% final concentration EtOH were added to the upper well and TER measured at the indicated time points. Activation conditions were performed in triplicate and shown is the result of one of three similarly performed experiments. (c) Caco-2 cells were grown and activated as in (a) but with the addition of FITC-labelled dextran beads (5 kDa size) into each upper chamber. At indicated time points, sample aliquots were taken from the lower trans-well chambers and the amount of transmigrated beads measured. Data shown are mean ± SD of three experiments. p-values derive from one-way ANOVA test and were assessed by Bonferroni post-test for multiple comparisons. (d) Experiments were performed as under (c) but with the addition of J96 bacteria instead of dextran beads into the upper chamber. Data shown are mean ± SD of two similarly performed experiments.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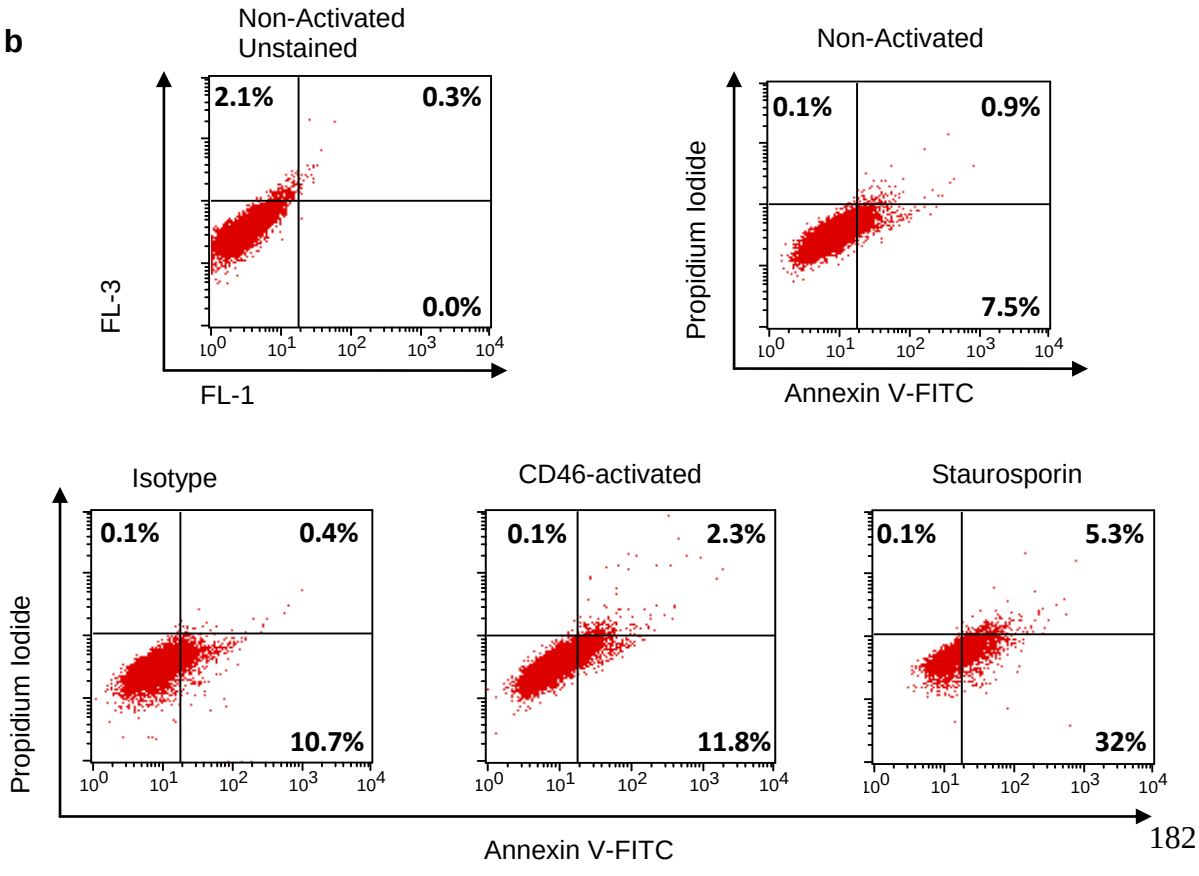
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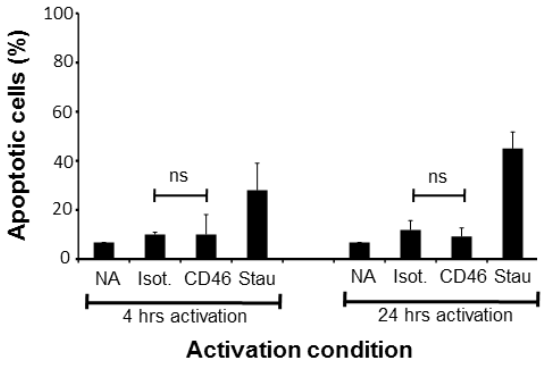

Figure 5.3 CD46 activation does not induce apoptosis in Caco-2 cells. Caco-2 cells were seeded at 1 x 10^5 cells per well and cultured in 24-well plates for 6 days. At day 6 cells were either left non-activated or treated with mAb to CD46 (10 µg/ml), isotype control mAb (IgG1; 10 µg/ml) or staurosporin (200 µM), an apoptosis-inducing agent. Apoptosis was analysed by combined Annexin-FITC and propidium iodide staining at 4 and 24 hours post activation. a) FACS plots relative to unstained non-activated Caco-2 cells (top left), non-activated stained cells (top right), isotype treated (lower left), anti-CD46 activated (lower centre) and staurosporin treated cells at 4 hours (lower right). b) FACS plots relative to cells either non-activated or activated as in (a) and stained at 24 hours. c) Summary of the percentage of apoptotic cells (Annexin-FITC positive, PI-) for four similarly performed experiments. Data shown are the mean ± SD and representative of four experiments. NA, non-activated; Stau, staurosporin; ns, statistically not significant as evaluated by Friedman's test with Dunn's multiple comparisons.

5.5 CD46 activation promotes cell growth and accelerates wound healing in intestinal epithelial cells

Proliferation of epithelial cells is controlled by contact inhibition. In this process, the formation and constellation of TJ and AJ eventually induces growth stop while a decrease in TJ and/or AJ or impairment of their integrity initiates cell proliferation (Zeng and Hong, 2008). Therefore CD46-mediated modulation of TJ and AJ formation on Caco-2 cell proliferation was assessed. Caco-2 cells were activated with either CD46 mAbs, an isotype-specific control mAb or treated with staurosporin for 24 or 48 hours and then assessed for cell proliferation (Figure 5.4a). Whilst staurosporin treatment induced cell death (as expected), CD46-activated cells showed a significant increase in proliferation compared to the control mAb-treated cultures.

The effect of CD46-mediated signals on epithelial cell growth was confirmed using a wound healing assay. Caco-2 cells were cultured on 24-wells with a removable insert that created a defined 'wound-like' area upon removal (Figure 5.4b, photographs). CD46 engaged *via* the addition of mAbs to CD46 and cell growth monitored *via* time-lapse microscopy over 18 hours. In agreement with the data obtained in the proliferation assay (Figure 5.4a), CD46 engagement accelerated would closure significantly compared to the addition of isotype control mAb (Figure 5.4b, lower graph).

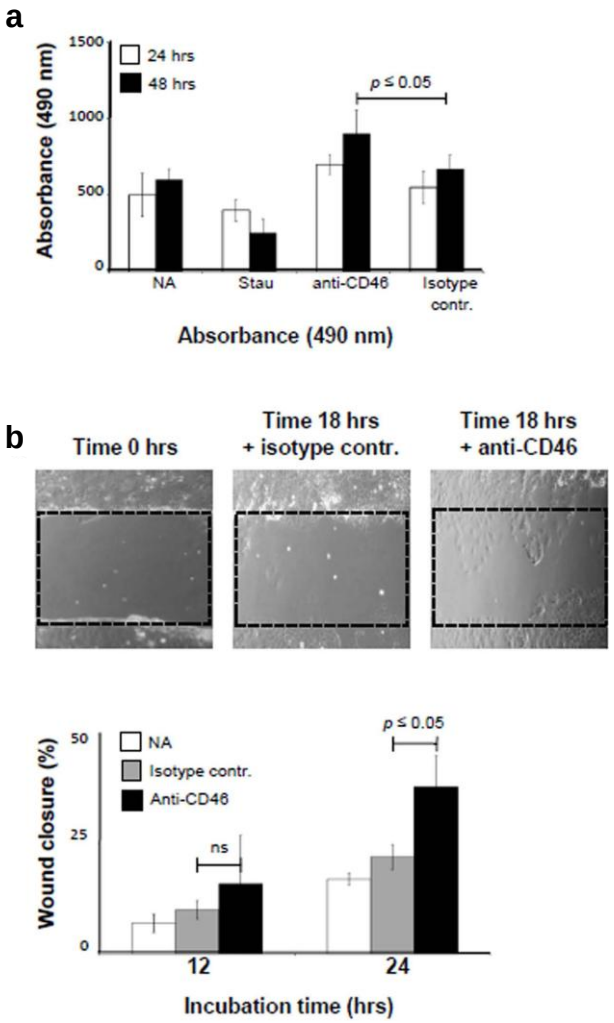

Figure 5.4 CD46 activation accelerates wound healing. (a). Caco-2 cells were seeded into 24-wells at 1.5 x 10^4 cells/well and allowed to adhere overnight. Wells were then left untreated or treated with 10 μg/ml mAb to CD46, an isotype control mAb or staurosporin (200 μM) and cell proliferation measured 24 and 48 hrs post treatment. Data shown are the mean ± SD and representative of four experiments. (b) Caco-2 cells were cultured on 24-wells with a removable insert. After 6 days, the insert was removed and revealed a defined 'wound-like' area (upper panels). mAbs to CD46 or an isotype control Ab was added and cell growth monitored via time-lapse microscopy. Lower panel shows the statistical analysis using MetaMorph of the data obtained visually. Data shown are the mean ± SD and representative of three experiments. ns, statistically not significant.

5.6 Discussion

Utilizing Caco-2 *in vitro* trans-well and culture systems, CD46 activation was found to reduce transepithelial resistance and increased paracellular permeability of intestinal epithelial cell monolayers (Figure 5.2). However, though CD46 activation allowed for an increased transmigration of pathogenic bacteria across the cell layer, it also induced increased cell proliferation and accelerated wound healing (Figure 5.3). These data suggest that CD46 plays a previously unacknowledged role in the regulation of epithelial cell barrier integrity and repair. These findings would be fully in line with an expected protective role of the complement system at such vital host/environment interface. Although in the *in vitro* systems used CD46 was engaged though a mAb, such engagement *in vivo* is easy to envisage. The intestinal lumen contains high amounts of C3 and C3b and several types of epithelial cells secrete C3 (which is then converted to C3b) upon activation by cytokines, tissue damage or other stress signals (Moon et al., 1997). Thus, CD46 activation at this location can easily be envisioned in an *in vivo* setting. It is important to notice that the engagement of CD46 in the data provided in this chapter was performed using a monoclonal antibody to CD46. The strength of such signal is deemed to be greater than the one of the physiological ligand of CD46, C3b. The use of C3b as a stimulus in this set of experiments could have led to different results for two main reasons: -the engagement may not trigger the same level of signalling strength as the monoclonal antibody –C3b may engage other receptors (e.g. CR1) or complement regulators (Factor H) which in turn allow for cleavage of C3b in smaller fragments (e.g. iC3b, C3dg and C3f) which can bind other complement receptors (e.g. CR2) rendering difficult to discriminate the role of CD46 engagement in epithelial cells from the engagement of other receptors. The observation that the addition of mAbs to CD46 decreased epithelial barrier was unexpected – specifically in the presence of pathogenic bacteria as one would content that ‘sensing’ of danger by a complement component would rather increase barrier function to protect the host from infection. The data presented in this chapter are in good agreement with the observation that mice transgenic for human CD46 show increased transmigration of pathogenic *Neisseria* through the blood brain barrier and subsequent high infection and mortality rate (Johansson et al., 2005). In fact, mice do not express CD46 on somatic cells (Inoue et al., 2003) and are usually not susceptible to pathogens targeting CD46 as cell entry receptor (Kemper et al., 2005). The finding though that

CD46 activation also increased cell proliferation and wound healing suggests that CD46's normal functional role on intestinal epithelial cells may rather be homeostatic *via* the induction of barrier repair after pathogen breach or other injury (and that some pathogens plainly take advantage of this 'house-keeping' function). The induction of epithelial cell proliferation and repair mechanism are induced *via* changes in TJ and AJ formation and signalling events triggered by those changes. Although the underlying mechanism is undefined, a vital role for complement activation in the repair after liver injury has previously been proposed as mice deficient for C3 and/or C5 fail to initiate hepatocyte proliferation and tissue repair in a liver injury model (He et al., 2009). Accordingly, patients with liver cirrhosis and impairment of tissue repair present with very low levels of circulating C3 and C4 (Nakagawa et al., 1998). A better understanding of the exact mechanism(s) by which CD46 regulates TJ and/or AJ formation or activation will now require an assessment of the CD46-mediated signalling events post activation in these cells. There are several possibilities as to how CD46 could participate in barrier homeostasis. CD46-mediated signals could impact (possibly *via* SPAK and/or α-E-catenin) on the expression level of TJ and AJ proteins. Changes in E-cadherin expression are generally connected with AJ functional changes (Ayollo et al., 2009). Interestingly, this has also been observed for changes in α-E-catenin and SPAK levels or functions (Kobielak and Fuchs, 2004). Studies of CD46-mediated impact in the expression of TJ proteins such as occludins and claudins have so far not been performed but will likely unravel also a cross-connection or -regulation axis as both TJs and AJs need to undergo modifications for TER changes. As an interesting third option (and these models may not be mutually exclusive), there could a potential crosstalk between CD46 and the Notch and/or Wnt system in intestinal epithelial cells. This idea is based on recent observations showing that CD46 regulates the expression of Notch 1 and 2 as well as their ligands jagged-1 and delta-1 in human $CD4^+$ T cells (Le Friec and Kemper, 2010; Cope et al., 2011). A role for CD46 in the regulation of barrier function and growth could have major implications for several disease states. For example, disturbance in CD46-mediated TJ and AJ function (*via* mutations in CD46 leading to suboptimal or excessive signalling) could induce 'leakage' of the gut flora from the lumen into the surrounding tissue and may cumulate into chronic inflammation such as observed in IBD (Hugot et al., 2001). This 'phenotype' may be further potentiated by an impairment of CD46-mediated

tissue/barrier repair. On the other hand, increased or uncontrolled induction of CD46-mediated cell proliferation may induce malignant transformation of epithelial cells and adenocarcinoma formation. Indeed, a mutation in CD46 that retains expression, complement regulatory and ligand binding function but induces a non-sense mutation in the intracellular domains has been identified in a colon cancer patient (Sjoblom et al., 2006). In summary, these data are in further support of the increasing understanding that complement is not only a pillar in the defence against invading pathogens and the shaping of the adaptive immune response, but also a key player in immune homeostasis and tissue repair.

Chapter 6

Conclusions and Future Directions

The identification of CD46 as a unique co-receptor for the TCR in the induction of IL-10 production was the first evidence bridging human complement to T cell-mediated immunosuppression describing an interesting further link between innate and adaptive immunity (Zaffran et al., 2001). The ability of complement-induced T cells (*via* CD46) to suppress bystander $CD4^+$, $CD8^+$ (Kemper et al., 2003; Capasso et al., 2006) and γδ T-cells (Truscott et al., 2010) raised controversy in regards to the paradigmatic role of complement activation. In fact, as complement is firstly a pathogen clearance system with broad properties, the identification of how it negatively regulates adaptive immunity and how it could be approached therapeutically was and still is a major challenge for immunologists.

The data presented in this thesis added knowledge to the field of T cell regulation via complement signals introducing the novel concept of complement-mediated T_H1 "switching". It unified previous controversial findings on the role of CD46 in $CD4^+$ T lymphocytes which reported either the induction of a predominant immunoregulatory phenotype (Kemper et al., 2003) or a T_H1 biased differentiation of $CD4^+$ T (Sanchez et al., 2004). As summarized in the model represented in figure 3.16, the findings in this thesis showed that cross-linking CD46 on the surface of $CD4^+$ T cells does not induce directly an immunosuppressive Tr1-like phenotype but rather initiates a more complex process of temporally regulated cytokine production. In this process engagement of CD46, which may occur via C3b, pathogen interaction or mAb cross linking (Kemper et al., 2003; Price et al., 2005), in the presence of low concentrations of IL-2 (i.e. resembling the initiation phase of an immune reaction) induces a T_H1 response with correspondent IL-2 and IFN-γ production. In the presence of high micro-environmental IL-2, which would be characteristic of the immune effector phase, the signaling through CD46 drives the switch of T_H1 cells into an IL-10 (Foxp3-) phase with regulatory properties. Such switch model clarifies how complement activation would primarily favour an immune response in the initial phase (CD46 induction of T_H1 cells) of an infection, and then modulate it so to re-establish a normal immune homoeostasis during the IL-10 phase. Hence, the findings in Chapter 3 and 4 highlighted how, in the contest of CD46 induced T cells, the implementation of signal 1 (TCR) signal 2 (CD46) and signal 3 (IL-2) results in the switching of T_H1 to Tr1 cells highlighting a novel pathway of T helper cell auto-regulation.

Since the identification of Tr1 cells 18 years ago (Bacchetta et al., 1994; Groux et al., 1997) no transcription factor has been associated to this immunoregulatory class of lymphocytes. Hence, their cellular origin, tissue location and function cannot be ascertained *in vivo* or *ex vivo* limiting the knowledge regarding the involvement of Tr1 lymphocytes in health and disease. Although CD46 activated lymphocytes were initially defined as Tr1-like T cells, the findings described in this thesis support the theory that IL-10 secreting cells may not necessarily represent a distinct T cell lineage. The data presented in Figure 3.4 clearly showed that the IL-10-secreting cells induced *via* TCR, CD46 and IL-2 signals do not generate from the expansion of an existing regulatory population. These results are in accordance with the accumulating set of data showing that IL-10 production from $CD4^+$ T cells can arise, according to environmental cues, from different T_H cell lineages including T_H2, T_H9 and T_H17 cells. Thus the T cell auto-regulation pathways may not be restricted to T_H1 lineages (McGeachy et al., 2009; Murphy and Stockinger, 2010). The data presented show how CD46-induced T cells are not a distinct lineage of Tr1 cells but rather represent the end point of a T_H1 response. Whether IL-10-secreting T_H1 cells and Tr1 cells represent different T cell lineages of a common precursor that has implemented micro-environmental cues differently is not a question that to date can be addressed given the absence of a marker for Tr1 identification.

Similarly, assessing the *in vivo* importance of CD46-mediated T cell regulation is equally difficult as rodents do not express CD46 on somatic cells and a suitable small animal model is therefore not available. However, data presented in Chapter 3 support that an *in vivo* role for CD46 exists in human. The observations showed for the first time that CD46-induced T_H1 cells are defective in RA. This is in accordance with previous findings regarding an altered CD46 immunoregulatory function in autoimmune diseases (Astier et al., 2006; Le Buanec et al., 2011). However, the defect in RA rather related to the inability in shutting down the T_H1 phase that consequently tipped the balance of IFN-γ *versus* IL-10 production in drastic favor of IFN-γ. These findings, taken together with unpublished results showing that T cells from RA in remission are able to correct this defect and revert to normal cytokine secretion, have at minimum two implications: 1. identifying key molecular clues in the shutdown of IFN-γ may provide a viable option for therapeutic translational applications since this seems to be a revertible defect *in vivo*. 2. IL-10-secreting T cells are able to revert *in*

vitro to IFN-γ production highlighting that a more detailed study of the pathways and epigenetic modifications involved is necessary in order to prevent a deleterious production of inflammatory cytokines in an attempt to ameliorate an autoimmune or inflammatory disease.

A further key point when considering the therapeutic potential of CD46-activated T cells is related to local complement production. Data generated from several groups (Heeger et al., 2005; Lalli et al., 2007; Peng et al., 2006; Pratt et al., 2002; Strainic et al., 2008) highlight immune-derived complement as a key player in the shaping of T cell immunity. Hence the development of complement drugs aimed towards the amelioration of autoimmune disorders, graft acceptance or inflammatory diseases must take into consideration this source of complement components. Tissue/organ delivered, cell-permeable complement inhibitors may be more effective than systemic inhibitors and pose lower risks in terms of infection related contraindications.

The data reported in Chapter 4 identified the protease AEP as a key player in the CD46 mediated switch at a molecular level. AEP has been highly investigated following its discovery in 1997 (Chen et al., 1997) mainly in APCs. The data generated in this thesis add to the knowledge of the field since it shows AEP expression and modulation in $CD4^+$ lymphocytes with an important functional consequence on the secretion of pro-inflammatory cytokines. In the bigger picture of the CD46-mediated switch, AEP is an important molecular target. Since inhibiting this protease clearly affects secretion of T cell derived IFN-γ, it could be argued that in a setting of autoimmune disease, where excessive IFN-γ is an important issue, targeting AEP and blocking its activation in the site of inflammation could potentially be beneficial. In the case that further work on AEP knockout mice would reinforce these findings, an animal model to assess AEP function in T_H1 cytokine secretion could be a viable option and answer the question of whether AEP targeting for amelioration of autoimmune diseases can be achieved.

The findings in Chapter 5 described an unacknowledged role for CD46 in intestinal epithelial wound healing reporting a novel role for complement in this cell type. Complement has previously been shown to be important in processes of wound repair. Several reports have shown that deficiency of complement components C3 and C5 is associated to impairment of hepatocyte regeneration in models of hepatic injury (He et al., 2009; Markiewski et al., 2004; Strey et al., 2003). Moreover, the central

complement component C3, can be produced by intestinal epithelium upon stimulation with pro-inflammatory signals (Moon et al., 1997). At the level of the gut, the production of complement components seems to be particularly important to maintain a state of tolerance. In fact, C3 deficient mice present with higher levels of pro-inflammatory cytokines and lower IL-10 in comparison to C3 sufficient mice (Pekkarinen et al.). Given the role of CD46 as a pathogen receptor, several studies have focused on the importance of CD46 engagement by pathogens at the level of epithelial tissues. For instance, CD46 interacts with *Neisseria meningitidis* and *Neisseria gonorrhoeae* and this interaction leads to different epithelial cell modifications including rearrangement of the cytoskeleton, calcium flux and bacterial uptake by epithelial cells (Crimeen-Irwin et al., 2003; Kallstrom et al., 1998; Lee et al., 2002). CD46 has also been described to be involved in the polarization of human epithelium. In fact, CD46 binds to the protein DLG4 in kidney epithelial cells and this interaction is important for cell polarization (Ludford-Menting et al., 2002). Hence, it is clear that CD46 engagement impacts epithelial cell biology. In this thesis the direct effects of CD46-activation through monoclonal antibodies, were investigated in intestinal epithelial cells. It was shown that Caco-2 monolayers cultured in a trans-well system have decreased trans-epithelial resistance when CD46 is engaged via antibodies. The reduction in trans-epithelial resistance was associated with an increase in paracellular permeability to dextran beads and pathogenic E. Coli. Importantly, although CD46 engagement decreased barrier permeability, it also increased cellular proliferation and accelerated the closure of a wound-like injury in an *in vitro* model of wound healing. Hence, these data highlighted an important role for CD46 in the regulation of epithelial cell barrier integrity and repair.

An important aspect which should be investigated in future studies is whether CD46 engagement induces the secretion of specific cytokines by epithelial cells. The complex cellular and cytokine environment of the gut has profound effects on the differentiation and function of the different cell types involved. It is known that intestinal epithelial cells are able to produce several cytokines in response to inflammatory stimuli, including; IL-1β, IL-6, IL-7, IL-8, TGF-β and chemokines such as IFN-γ inducible protein-10, IFN-inducible T cell alpha chemokine and CCL5 (Dignass and Podolsky, 1993; Dotan et al., 2007; Dwinell et al., 1999; Mazzucchelli et al., 1996; McGee et al., 1993; Seydel et al., 1997). Cytokines derived from IECs can

impact directly on neighbouring epithelial cells inducing differentiation and wound healing (TGF-β) or promoting epithelial cell survival (IL-6) (Beck et al., 2003; Jin et al., 2010; Kurokowa et al., 1987) but could also impact on bystander T cells promoting the production of pro-inflammatory or anti-inflammatory cytokines and/or the migration of T cells via the secretion of chemokines/chemoattractants (Pratt et al., 2002; Reinecker and Podolsky, 1995). Given the multiple functions of epithelial-derived cytokines it would be interesting to investigate whether CD46 stimulation of intestinal epithelial cells drives the production of chemokines and/or cytokines which could impact on both T cell and epithelial cell biology.

An additional aspect which requires further investigation is represented by the complement components produced by epithelial cells and their effects on bystander T cells. Several studies have highlighted the importance of epithelial-derived complement in the regulation of epithelial cell biology and in the modulation of T cell responses (Pekkarinen et al., 2012; Pratt et al. 2002). Although local complement induction has been mainly addressed in APCs and T cells, an elegant study by Pratt et al. (2002) firstly highlighted the importance of local complement production by epithelial cells and its effect on $CD4^+$ T cells in a transplant setting (Pratt et al., 2002). The authors showed that C3 deficient renal grafts had prolonged survival, in comparison to wild type renal grafts, when transplanted in C3 sufficient animals; at the same time they proved that a C3 sufficient graft did not have prolonged survival in C3 deficient recipients. The source of C3 was identified in the epithelial cells of the kidney and the authors showed that local C3 has an important immunomodulatory effect on T cells. In fact, stimulation of T cells in the presence of C3 deficient epithelial cells induced less IL-2 production than C3 sufficient epithelial cells. Hence, studying co-cultures of C3 sufficient and deficient intestinal epithelial cells and human $CD4^+$ T cells could shed light on whether C3 derived from intestinal epithelial cells impacts T cell biology and whether it induces a similar cytokine profile to CD46 activated of T cells.

CD46 mediated signals on T cells indeed up-regulate the expression of gut associated receptors (e.g. $\alpha_4\beta_7$ and CCR9) (Alford et al., 2008) and regulate the function of the kinase SPAK which was shown in this thesis to be a signalling molecule able to associate to CD46 in epithelial cells. These findings support the hypothesis of the gut

being a likely site where CD46 induced T cells may reside *in vivo*. This hypothesis would support a dual role of complement-mediated signals particularly at host/environmental interfaces, including the intestine, lung and skin. In fact, on one hand complement's inflammatory function would be necessary to prevent infection or clear infecting pathogens but on the other hand complement's regulatory function would be essential for the regulation of T cell responses and for the repair of tissue injury accompanying the inflammatory response. This role for complement-mediated signals is supported by the gene array data from CD46-induced T cells showing mainly the expression of inflammatory related genes in the T_H1 phase induced by CD46 but also the expression of wound healing genes (e.g. metalloproteinases for tissue remodelling) in the contraction phase. Hence in future studies supernatants from CD46 mediated IFN-γ+/IL-10(-), IFN-γ+/IL-10+ and IFN-γ(-)/IL-10+ populations should be prepared and tested on epithelial cultures to evaluate their ability to promote epithelial wound repair. Moreover, the sorted three populations might be used in a co-culture system with epithelial cells and DCs to attest for DCs influence on cytokine production and wound healing. Vaknin-Dembinsky showed how IL-23 increased expression by myeloid DCs activated through CD46 was able to induce IL-17 secretion by T cells (Vaknin-Dembinsky et al., 2008). It would be interesting to evaluate the role of CD46 in intestinal DCs and add a level of complexity to the system by co-culturing T cells, DCs and epithelial cells. Additionally, since AEP is expressed in mice the development and investigation of the role of T_H1 cells in an *in vivo* model of wound healing could add knowledge to the understanding of the complex networks of signals that are implicated during the clearing of an intestinal infection.

In summary, this thesis further supports the following two emerging new paradigms in the complement field 1- complement is required for tissue and immune homeostasis and 2- cell-derived local complement activation vitally shapes adaptive immune responses.

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Analyte: IFNg

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	275	4.47	54.62%	0.00	0.00	-	0.00
Std02	20.00	253	8.56	39.57%	1.30	1.32	1.69%	21.04
Std03	40.00	232	12.22	43.08%	1.60	1.60	0.16%	39.77
Std04	80.00	210	19.17	44.51%	1.90	1.88	1.06%	76.36
Std05	156.00	208	32.98	61.70%	2.19	2.17	0.85%	149.47
Std06	312.50	232	66.93	49.49%	2.49	2.51	0.81%	327.33
Std07	625.00	240	136.62	466.07%	2.80	2.84	1.52%	689.46
Std08	1250.00	235	221.67	82.73%	3.10	3.06	1.34%	1135.69
Std09	2500.00	247	460.38	68.80%	3.40	3.39	0.28%	2446.43
Std10	5000.00	274	904.80	112.89%	3.70	3.71	0.38%	5162.87

Fitting Equation

5 parameter logistic: log I = D + (A - D) / (1 + ((log CC - E) / C)^B)
Parameters: A=0.65, B=3.00, C=4.04, D=5.32, E=-0.29
Fitting accuracy: R square = 99.92%

Analyte: IL-10

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	235	4.73	71.84%	0.00	0.00	-	0.00
Std02	20.00	258	30.62	30.89%	1.30	1.30	0.10%	19.94
Std03	40.00	241	56.25	26.88%	1.60	1.62	0.98%	41.47
Std04	80.00	240	97.66	32.27%	1.90	1.88	1.23%	75.81
Std05	156.00	174	200.69	30.19%	2.19	2.20	0.38%	159.01
Std06	312.50	236	391.42	87.96%	2.49	2.49	0.17%	309.46
Std07	625.00	255	812.12	28.58%	2.80	2.81	0.38%	640.40
Std08	1250.00	243	1566.88	28.93%	3.10	3.10	0.04%	1253.75
Std09	2500.00	254	2915.38	32.52%	3.40	3.39	0.33%	2436.33
Std10	5000.00	226	5522.16	21.02%	3.70	3.70	0.13%	5056.76

Fitting Equation

5 parameter logistic: log I = D + (A - D) / (1 + ((log CC - E) / C)^B)
Parameters: A=0.68, B=3.55, C=5.12, D=6.04, E=-1.85
Fitting accuracy: R square = 99.99%

Analyte: IL-2

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	264	6.99	35.26%	0.00	0.00	-	0.00
Std02	20.00	281	35.52	24.68%	1.30	1.31	0.38%	20.23
Std03	40.00	296	63.36	27.14%	1.60	1.62	0.81%	41.22
Std04	80.00	283	110.40	30.55%	1.90	1.88	1.46%	75.04
Std05	156.00	284	244.82	27.82%	2.19	2.22	1.10%	164.88
Std06	312.50	265	456.25	114.28%	2.49	2.47	0.84%	297.76
Std07	625.00	288	990.05	37.77%	2.80	2.80	0.02%	624.04
Std08	1250.00	290	2071.08	25.31%	3.10	3.12	0.65%	1309.40
Std09	2500.00	295	3717.03	23.60%	3.40	3.39	0.11%	2477.81
Std10	5000.00	294	6551.49	21.16%	3.70	3.69	0.17%	4929.89

Fitting Equation

5 parameter logistic log I = D + (A - D) / (1 + ((log CC - E) / C)^B)
Parameters: A=0.85, B=5.70, C=6.03, D=5.18, E=-3.21
Fitting accuracy: R square = 99.97%

Analyte: IL-4

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	309	7.98	35.89%	0.00	0.00	-	0.00
Std02	20.00	303	28.43	29.55%	1.30	1.30	0.05%	20.03
Std03	40.00	273	49.03	31.90%	1.60	1.61	0.49%	40.73
Std04	80.00	298	84.44	30.66%	1.90	1.88	1.28%	75.64
Std05	156.00	221	176.83	29.44%	2.19	2.21	0.94%	163.59
Std06	312.50	321	341.89	30.91%	2.49	2.50	0.21%	316.33
Std07	625.00	305	642.57	29.36%	2.80	2.77	0.79%	594.05
Std08	1250.00	305	1381.37	29.27%	3.10	3.12	0.64%	1308.69
Std09	2500.00	312	2435.23	28.15%	3.40	3.39	0.33%	2436.56
Std10	5000.00	321	4490.01	23.30%	3.70	3.70	0.09%	5039.11

Fitting Equation

5 parameter logistic log I = D + (A - D) / (1 + ((log CC - E) / C)^B)
Parameters: A=0.90, B=3.30, C=4.23, D=5.60, E=-1.00
Fitting accuracy: R square = 99.97%

Analyte: IL-5

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	236	5.44	63.48%	0.00	0.00	-	0.00
Std02	20.00	208	55.74	32.66%	1.30	1.31	0.88%	20.54
Std03	40.00	245	100.82	32.15%	1.60	1.61	0.33%	40.49
Std04	80.00	229	180.06	30.28%	1.90	1.87	1.57%	74.67

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std05	156.00	209	387.91	32.90%	2.19	2.21	0.63%	161.06
Std06	312.50	251	762.51	31.44%	2.49	2.50	0.02%	312.79
Std07	625.00	264	1511.47	33.89%	2.80	2.79	0.19%	617.55
Std08	1250.00	254	3090.99	29.62%	3.10	3.11	0.51%	1296.18
Std09	2500.00	240	5522.16	31.14%	3.40	3.39	0.20%	2461.91
Std10	5000.00	254	9909.46	13.07%	3.70	3.70	0.07%	4968.90

Fitting Equation

5 parameter logistic log I = D + (A - D) / (1 + ((log CC - E) / C)^B)
Parameters: A=0.74, B=5.05, C=6.47, D=5.76, E=-3.61
Fitting accuracy: R square = 99.98%

Analyte: TNF

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	229	4.38	56.08%	0.00	0.00	-	0.00
Std02	20.00	251	9.00	35.62%	1.30	1.31	0.93%	20.57
Std03	40.00	255	12.95	32.86%	1.60	1.59	0.96%	38.61
Std04	80.00	263	20.10	31.09%	1.90	1.86	2.17%	72.76
Std05	156.00	201	40.42	28.26%	2.19	2.23	1.75%	170.44
Std06	312.50	270	71.99	27.07%	2.49	2.50	0.20%	316.12
Std07	625.00	261	161.53	30.90%	2.80	2.84	1.62%	693.67
Std08	1250.00	284	267.96	27.78%	3.10	3.04	1.80%	1099.17
Std09	2500.00	250	672.17	29.39%	3.40	3.39	0.35%	2432.97
Std10	5000.00	244	1699.08	35.63%	3.70	3.72	0.52%	5227.87

Fitting Equation

5 parameter logistic log I = D + (A - D) / (1 + ((log CC - E) / C)^B)
Parameters: A=0.64, B=2.22, C=6.88, D=13.39, E=-0.00
Fitting accuracy: R square = 99.86%